The effect of maize vitreousness and a starch binder on *in vitro* fermentation parameters and starch digestibility in dairy cows

by

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at

Stellenbosch University

Department of Animal Sciences

Faculty of AgriScience

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Date: *December* 2017

DECLARATION

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the authorship owner thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: December 2017

Johan Hendrik Combrink van Zyl

Abstract

Title	:	The effect of maize vitreousness and a starch binder on *in vitro* fermentation parameters and starch digestibility in dairy cows
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Maize kernels consist of hard vitreous endosperm and soft floury endosperm, and the ratio of the vitreous to floury endosperm determines the vitreousness of the kernel. As ruminal fermentation and animal performance are higher for low vitreous maize, lower vitreous maize is favoured for inclusion in animal feeds. Very high ruminal starch degradability may, however, lead to metabolic disorder risks such as acidosis. The objective of this study was to evaluate various techniques for routine analysis to determine a rapid, simple, inexpensive method to predict maize vitreousness accurately. Secondly, the usability of Near-infrared spectroscopy (NIR) technology in the anmal feed industry to predict the fractional rate and extent of ruminal starch degradability in maize differing in vitreousness was investigated by means of *in vitro* starch disappearance. Thirdly, the effect of the treatment of maize with a commercial starch binder on rumen kinetics of lactating dairy cows was investigated by means of *in vitro* gas production and *in vitro* starch disappearance studies. For this part of the study, it was attempted to bind some of the maize starch (1 mm grind) *in vitro* with the treatment of a commercial starch binder. The fourth objective was to investigate the effect of particle size (1mm vs 4 mm grind) and a starch binder on *in vitro* disappearance of starch in low vitreous maize. The final objective was to determine the effect of starch binder treatment of low vitreous maize on the apparent total tract digestibility and production responses in lactating dairy cows.

Ninety maize samples of different vitreousness were collected and subjected to NIR at a single absorbance of 2230 nm and PSI trough a single 106 µm screen. Samples were subsequently ranked according to vitreousness. The ten hardest and ten softest samples were selected to evaluate NIR, particle size index (PSI), and Rapid visco analyzer (RVA) rheological analyses as potential methods for the determination of maize vitreousness against X-ray micro-computed tomography (XCT). Significant

relationships were found between NIR and PSI regarding hardness predictions, while the study with the smaller sample set (n = 10) showed significant relationships between PSI, NIR, RVA peak time (corresponding time required for a sample subjected to rheological analysis to reach peak viscosity) and RVA peak viscosity (the process of gelatinization and occurs at the equilibrium point between swelling and polymer leaching) in relation to XCT regarding maize vitreousness determination. All other rheological information data were not accurate to predict maize hardness. As NIR technology is already available and meets the requirements of speed, simplicity and inexpensiveness, it was concluded that NIR at a single absorbance of 2230 nm is the most accurate and practical method to determine maize vitreousness in the animal feed industry.

Thereafter, six maize samples of decreasing vitreousness were selected from ninety samples with known vitreousness and subjected to *in vitro* starch disappearance at 0, 3, 6, 12, 24 and 48 h of incubation. The subsequently determined fractional rate of disappearance and predicted ruminal starch disappearance decreased significantly as maize vitreousness increased. Hardness indexes calculated from NIR analyses at a single absorbance of 2230 nm showed inverse linear and quadratic relationships for both fractional rate and extent of starch disappearance. It was concluded that NIR technology could be used to predict fractional rate and extent of starch disappearance from the rumen based on maize vitreousness.

In a further study one low and one high vitreous maize sample were selected from the ninety samples with known vitreousness. Both samples were treated with equal amounts of a commercial starch binder (Bioprotect) and distilled water to determine the effect of the treatment on *in vitro* gas production and *in vitro* starch disappearance. The rate of *in vitro* gas produced from low vitreous maize was higher than that of high vitreous maize, irrespective of treatment. All other *in vitro* gas production parameters did not differ between treatments. *In vitro* starch disappearance values at 6, 12 and 24 h time intervals were, irrespective of binder treatment, higher with low vitreous maize compared to high vitreous maize. Starch binder treatment, however, did not affect *in vitro* starch disappearance.

In a further *in vitro* study, maize samples of known low vitreousness were milled through 1 mm and 4 mm sieves, respectively. The milled samples were then thereafter treated with equal amounts of a starch binder (Bioprotect) and distilled water to determine the effect of particle size and treatment on *in vitro* starch disappearance after 0, 3, 6, 12, 24 and 48 h of incubation. Particle size reduction increased ($P < 0.05$)

both fractional rate and extent of starch disappearance, while binder treatment showed a tendency ($P < 0.10$) towards decreased fractional rate and extent of starch disappearance. Despite no differences in ruminal kinetics with the binder treatment of 1 mm milled maize, treatment of 0.4 mm milled maize, however, indicated lower ($P < 0.05$) fractional rate and extent of starch disappearance. It was concluded that a reduction in particle size of maize with hammer mill processing changes rumen starch fermentation characteristics and that the treatment of 4 mm milled maize with a commercial starch binder may alter rumen fermentation kinetics.

In the final trial, six primiparous Holstein dairy cows were used to investigate the effect of a starch binder (Bioprotect) treatment of low vitreous maize on total tract nutrient digestibility and production parameters of lactating dairy cows. Starch binder (10/L/tonne grain) or water treated maize were used in two TMR's. No differences in dry matter intake, milk yield, 4% fat corrected milk yield, energy corrected milk yield, milk fat concentration, milk fat yield, milk protein concentration, milk protein yield, milk urea nitrogen concentration or somatic cell count were found between binder treated or water treated maize. Apparent estimated ruminal pH and the ratio of milk protein (%) to milk fat (%) also did not differ between treatments. Although total tract dry matter and nitrogen digestibilities did not differ between treatments, total tract starch digestibility decreased ($P < 0.05$) when maize was treated with a starch binder compared to the water treatment. It was concluded that the commercial starch binder might not be an effective tool to manipulate total tract maize starch digestion in dairy cows, as is apparently the case with wheat and barley. However, when the prevention of acidosis in dairy cows that receive high amounts of low vitreous maize is the objective, then a starch binder may prove to be effective.

Uittreksel

Titel	:	Die invloed van mielie-hardheid en ‘n styselbinder op *in vitro* fermentasieparameters en styselverteerbaarheid in lakterende melkkoeie
Kandidaat	:	Johan Hendrik Combrink van Zyl
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Graad	:	PhD in Veekunde

Mieliepitte bestaan uit beide harde- en sagte endosperm en die verhouding van harde tot sagte endosperm, bepaal mielie-hardheid. Weens verhoogde ruminale verteerbaarheid en diereprestasie met sagte- teenoor harde mielies, word sagte mielies in herkouerdiëte verkies. ‘n Baie hoë ruminale stysel-fermentasietempo kan egter tot metaboliese steurnisse, soos assidose, lei. Die doelwit van hierdie studie was om verskillende tegnieke te ondersoek om ‘n vinnige, eenvoudige en koste-effektiewe metode te vind om mielie-hardheid akkuraat, te bepaal. ‘n Tweede doelwit was om die doeltreffendheid van die gebruik van NIR (Near-infrared spectroscopy) skandering vir vinnige, akurate voorspellings van die tempo- en hoeveelheid van ruminale styselverdwyning in mielies met te ondersoek. ‘n Derde doelwit was om die invloed van die behandeling van mielies met ‘n styselbinder op rumenwerking in lakterende melkkoeie deur middel van *in vitro* gasproduksie en *in vitro* styselverteerbaarheid te ondersoek. ‘n Poging om van die mieliestysel deur die behandeling met ‘n kommersiële styselbinder te bewerstellig is dus ondersoek. Hierna is die *in vitro* styselverteerbaarheid van mieliestyselbinder-behandeling tesame met verkleining van partikelgroote deur maal met ‘n hammermeul (1 mm vs. 4 mm) van sagte mielies ondersoek. ‘n Finale doelwit van hierdie studie was om *in vivo* te bepaal of die behandeling van sagte mielies met ‘n styselbinder ‘n invloed op totale nutriënt-verteerbaarhede en diereproduksie het.

Negentig mieliemonsters met verskillende grade van hardheid is versamel en deur ‘n NIR met ‘n enkele absorpsie van 2230 nm geskandeer. Siffraksie-ontledings deur ‘n enkele 106 µm sif is ook gebruik om mielie-hardheid te bepaal. Vanuit hierdie data is tien harde en tien sagte monsters geselekteer vir verdere NIR-, sif (particle size index

(PSI))- en RVA (Rapid visco analyzer)-analises om akkuraatheid van mielie-hardheid toetsmetodes teenoor XCT (X-straal analisse) te bepaal. Betekenisvolle korrelasies om mielie-hardheid te bepaal is tussen die siffraksie, NIR, XCT, RVA piektyd en RVA piekviskositeit met beide datastelle bevind. Alle ander reologiese data kon nie mielie-hardheid akkuraat beskryf nie. Gesien in die lig daarvan dat NIR tegnologie nie net voldoen aan die behoeftes van die veevoerbedryf ten opsigte van tempo van ontleding, eenvoud en lae koste nie, maar ook aan die beskikbarheid van die tegniek, maak NIR die gewenste metode in die praktyk.

Ses mieliemonsters van dalende en bekende hardheid is geselekteer en vir 0, 3, 6, 12, 14 en 48 uur *in vitro* verteer. Berekende tempo- en mate van styselverdwyning was betekenisvol stadiger ($P < 0.05$) en minder namate mieliehardheid verhoog het. Betekenisvolle inverse liniêre en kwadratiese verwantskappe is tussen NIR skandering en beide die tempo- en mate van runimale styselverdwyning waargeneem. Die gevolgtrekking is gemaak dat NIR skandering effektief binne die veevoerbedryf gebruik kan word om die tempo- en mate van styselverdwyning in mielies met verskillende hardhede te voorspel.

Een harde- en een sagte mieliemonster, geselekteer uit die negentig monsters met bekende hardheid, is vir verdere studies geselekteer. Beide monsters is (na maal deur 1 mm sif) afsonderlik met gelyke dele van ‘n kommersïele styselbinder (Bioprotect) en gedistilleerde water behandel om die invloed van behandeling op *in vitro* gasproduksie en *in vitro* styselverteerbaarheid te bepaal. Die tempo van gasproduksie van sagte mielies, ongeag behandeling, was hoër teenoor die van harde mielies. Geen ander gasproduksieparameters het tussen behandelings verskil nie. Die 6, 12 en 24 uur *in vitro* styselverteerbaarhede van sagte mielies was, ongeag behandeling, hoër vir al drie tye teenoor harde mielies.

‘n Enkele sagte mieliemonster is deur 1 mm en 4 mm siwwe gemaal en daarna met gelyke dele styselbinder (Bioprotect) of gedistilleerde water behandel. Die verkleining van mieliepartikelgroote het die 0, 3, 6, 12, 24 en 48 uur *in vitro* tempo- en mate van styselverdyning betekeninsvol verhoog, terwyl ‘n neiging ($P < 0.10$) van verlaagde tempo en hoeveelheid van styselvertering met styselbinder-behandeling waargeneem is. Ten spyte van geen verskille in tempo- en hoeveelheid van styselverdwyning met styselbinder-behandelde mielies nie, het behandelde mielies ‘n betekenisvol laer tempo- en mate van styselverdyning getoon. ‘n Verlaging in mieliepartikelgroote met ‘n hammermeul verander dus ruminale parameters, terwyl 4 mm styselbinder-behandeling van sagte mielies ruminale fermentasieparameters kan verander.

Die invloed van die behandeling van sagte mielies met ‘n styselbinder (Bioprotect) op totale spysverteringskanaalverteerbaarhede en produksieparameters van ses eerstekalf lakterende Holsteinkoeie is bepaal. Beide styselbinder-behandelde (10L/ton graan), sowel as onbehandelde mielies, is in ‘n volvoerrantsoen aan die diere voorsien. Geen verskille tussen behandelings is in droëmaterialinname, melkproduksie, 4% vetgekorrigeerde melkproduksie, energiegekorrigeerde melkproduksie, bottervetinhoud en -opbrengs, melkproteïenkonsentrasie en -opbrengs, melk ureumstikstof en somatiese seltelling waargeneem nie. Daar is verder ook geen verskille in voorspelde rumen pH of die verhouding tussen melkproteïen- en bottervetinhoud tussen behandelings waargeneem nie. Ten spyte van geen verskille in totale spysverteringskanaal droëmateriaal- en stikstof verteerbaarhede nie, was totale spysverteringskanaal styselverteerbaarheid met styselbinder-behandelde 4 mm mielies betekenisvol laer ($P < 0.05$) teenoor die van onbehandelde mielies. Die gevolgtrekking is gemaak dat die kommersiële styselbinder waarskynlik nie ‘n doeltreffende hulpmiddel is om styselverteerbaarheid in melkkoeie te manipuleer, soos wat skynbaar die geval met koring en gars is nie. Indien die voorkoming van asidose by melkkoeie wat baie hoë vlakke van sagte mielies inneem egter die doelwit is, kan die behandeling van sulke mielies met ‘n styselbinder moontlik doeltreffend wees.

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List of Abbreviations

2-D:	Two-dimensional
3-D:	Three-dimensional
AA:	Amino acid
ADF:	Acid Detergent Fiber
AME:	Apparent metabolizable energy
AMTS:	Animal modeling and training systems
ANOVA:	Analysis of Variance
BW:	Body weight
CAT:	Computed Axial Tomography
CF:	Crude fiber
CO^2	Carbon dioxide
CP:	Crude protein
cP:	Viscosity
CT:	Computed Tomography
CV:	Coeficient of variance
dH_2O:	Distilled water
DIM:	Days in milk
DM:	Dry matter
DNA:	Deoxyribonucleic acid
ECM:	Energy corrected milk
EE:	Ether extract

EKV: Entire kernel volume

FE: Feed efficiency

FEV: Floury endosperm volume

GIT: Gastro-intestinal tract

HPC: High protein concentrate

H_2O: Water

ICC: Intraclass correlation

IDF: International Dairy Federation

iNDF: Indigestible neutral detergent fiber

k_d: Fractional rate of disappearance

MPO: Milk Producer Organization

MUN: Milk urea nitrogen

NaOH: Sodium hydroxide

NDF: Neutral detergent fiber

NFC: Non fiber carbohydrates

NFE: Nitrogen free extract

NIR: Near Infrared

NRC: Nutrient Research Council

NSC: Non structural carbohydrates

O_2 Oxigen

OM: Organic matter

PRD: Predicted ruminal disappearance

P:F: Milk protein concentration to milk fat concentration ratio

PSI:	Particle size index
RDS:	Ruminal degradable starch
REML:	Restricted maximum likelihood
RMO:	Rumen micro organisms
ROI:	Regions of interest
RPM:	Revolutions per minute
RPT:	Reading Pressure Technique
RVA:	Rapid Visco Analyzer
SAGL:	South African Grain Laboratory
SARA:	Sub-acute rumen acidosis
SCC:	Somatic cell count
TADD:	Tangential abrasion dehulling device
TMR:	Total mixed ration
TTDMD:	Total tract dry matter digestibility
TTND:	Total tract nitrogen digestibility
TTSD:	Total tract starch digestibility
V:F:	Vitreous to floury endosperm ratio
VEPAC:	Variance Estimation and Precision
VEV:	Vitreous endosperm volume
VFA:	Volatile fatty acid
XCT:	X-ray Micro-computed Tomography

Note

All literature were referenced according to guidelines of the Journal of Dairy Science. Instructions to authors. 2015. American Dairy Science Association. J. Dairy Sci. 98: Instructions 1-17.

CHAPTER 1

Introduction

1.1 General introduction

Maize (*Zea mays L.*) is, at a global production of almost 1,1 billion tonnes per annum during the year of 2014 (FOA, 2016), the largest cash crop produced internationally and by far the most widely used energy source in ruminant feed (Dihman *et al*., 2002; Lopes *et al*., 2009). Maize is grown in most countries and utilized as human food, animal feed and in ethanol production (Ranum *et al*., 2014).

1.2 Genetic modification

During the past two decades significant production progress has been achieved by genetic research and the development of modern maize cultivars (The maize trust, 2015). This has been achieved through genetic engineering (GMO) by genetically modified cultivars. Yield possibilities of modern maize cultivars have improved exponentially. Today cultivars that are resistant to drought, diseases and pests are used almost without exception. Currently 85% of all maize crops produced in South Africa are genetically modified (The maize trust, 2015). While significant grain production increases were achieved with GMO technology (Borlaug and Dowswell, 2003), often the impact of changed kernel morphology (in particular the endosperm) on ruminant digestibility was overlooked (Owens, 2005). The requirement to accurately describe maize endosperm characteristics on a routine basis within the animal feed industry therefore exists. Various methodologies to determine maize vitreousness exists and includes the particle size index (Burden, 2010; Cruywagen, 2016), Rapid Visco Analyser (Yamin *et al.*, 1999; Seetharaman *et al.*, 2001; Ji *et al.*, 2003; Sandhu & Singh, 2007), near infrared spectroscopy (Fox & Manley, 2009) and X-ray technology (Gustin *et al*., 2013; Guelpa, 2015). An accurate rapid, inexpensive and simple technique is, however, required for this routine analysis and to date does not exit.

1.3 Global maize production

Global maize production has reached the 1100 million ton per annum mark during 2014 (FAO, 2016).

Although production can be affected by climatic and other environmental conditions, 11 countries are producing 80% of global production. Figure 1.1 indicates the annual maize production distribution for 2014. Only two countries namely the United States of America and China produce more than 50% of the annual global maize production.

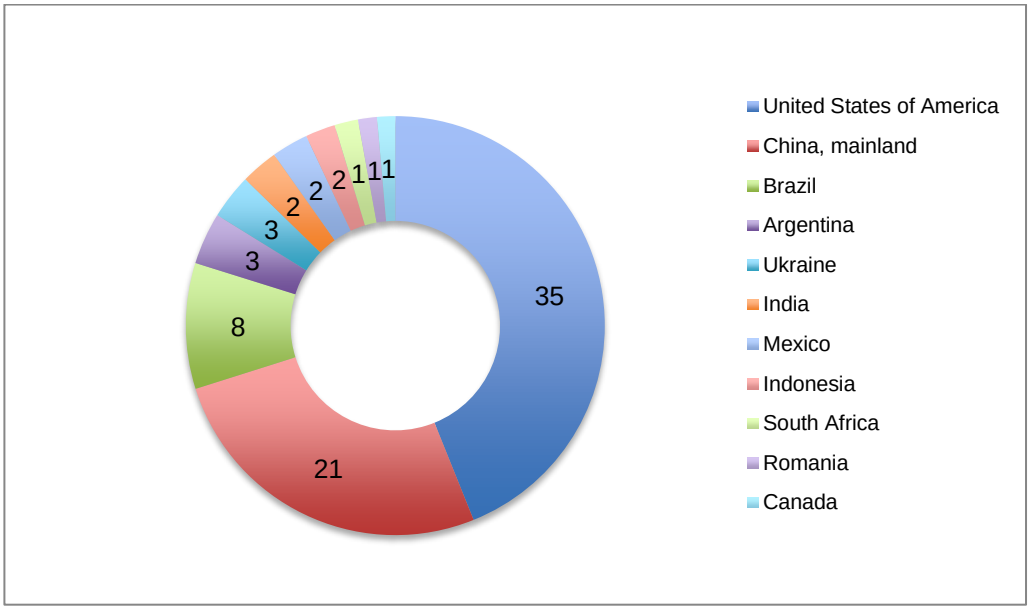

Figure 1.1. Global maize production contribution (%) per country for 2014 (FAO, 2016).

1.4 Global milk demand

Milk in either liquid or processed form (butter, yogurt, cream, ice cream, powdered milk) is a major source of nutrition to humans on a global scale. Figure 1.2 indicates total and per capita global production and consumption of dairy products. As can be seen from Figure 1.2, not only has the global population growth been mimicked by consumption, but also per capita consumption data indicate an increased demand for dairy products. This higher demand for dairy products (Figure 1.2) increases the pressure on dairy farmers to increase production via scaling their enterprises in size

to exploit economies of scale opportunities to supply the increasing demand.

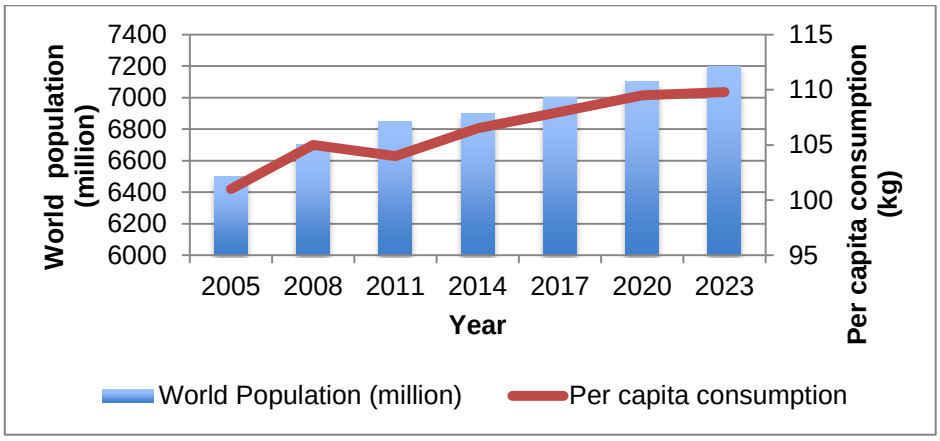

Figure 1.2. World population and per capita consumption of dairy products, 2005 - 2013 (IDF Bulletin, 2014).

In an effort to satisfy this increasing demand, animal productivity and increased production per animal is paramount and depends on raw material, including maize, optimization. This phenomenon occurs on a global scale. Figure 1.3 indicates the steady increase in raw milk produced in South Africa.

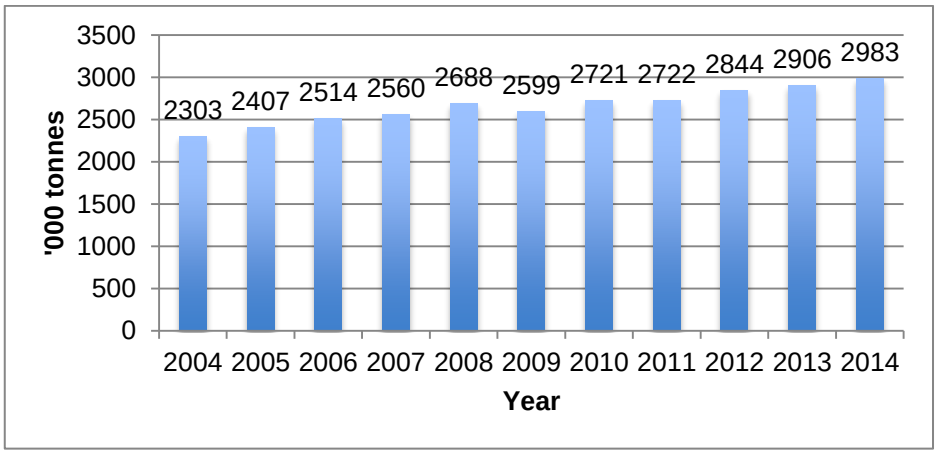

Figure 1.3. South African annual raw milk purchases, 2004 - 2014 (MPO, 2014).

1.5 From maize to feed to milk

Accurate nutrition is needed to increase and maintain high milk production, proper heath and high reproduction rates of the modern dairy cow (Van der Merwe & Smith, 1991). As feed cost is the most important input cost, it is crucial to optimize feed cost to ensure optimum profitability. Total feed cost comprises to 70-80% of a dairy farm's running cost (Muller, 2010). To ensure optimum milk production, various components of feed needs to be optimized. Protein, energy, fibre, minerals and vitamins are required in a balanced form to ensure optimum animal production. The main supply of energy to dairy cows fed a TMR is in the form of starch. Grain is the major form of starch in a TMR dairy diet. It is well documented that maize is by far the most widely used grain in dairy diets (Joy *et al.*, 1997; Shabi *et al*., 1999; Blasel, *et al*., 2006). As the pressure for food security increases in future, the pressure for optimal use of grain for animal feed is also expected to increase (Evers *et al*., 1999). The biggest component of maize is the endosperm that consists of both starch and protein. Starch is by far the larger of the two and is packed with nutrient rich cells to supply nutrients to support growth of the embryonic axis during germination (Evers *et al*., 1999). The biggest variation within maize is embedded in the shape and character of the endosperm.

Maize has a high metabolisable energy content ranging from 12.9 to 13.9 MJ ME/kg DM) value, is low in fibre (ADF ranges between 30 and 34 g/kg DM and NDF ranges between 90 and 95 g/kg DM) and contains about 730 g starch/kg DM (Van der Merwe & Smith, 1991; National Research Council, 2001; McDonald *et al.,* 2002). Starch provides approximately 75% of the energy of maize (Gencoglu, *et al*., 2009; Ranum *et al*., 2014). Generally maize has relative low protein and fat values of 85 g/kg to 90 g/kg DM and 35 g/kg to 40 g/kg DM, respectively (National Research Council, 2001; Ranum *et al*., 2014). Maize fed to ruminants is subject to the same advantages and disadvantages and risks as other cereals. The relatively slow digestion of maize in the rumen compared to other grains is an advantage and will reduce risk of acidosis in high producing ruminants fed large amounts of starch in the form of cereal grains. Rumen microorganisms (RMO) are mainly responsible for starch digestion in ruminants.

Whole grain, with an intact pericarp, is almost completely resistant to ruminal fermentation because microbes are unable to attach to the whole kernels (Callison *et al*., 2001; Eastridge *et al*., 2010).

Various processing techniques have been shown to increase both ruminal and total tract starch digestibility. Sodium hydroxide (NaOH) has been shown to enhance total tract starch digestion (Campeneere, *et al.,* 2006). Dry rolling, milling, steam flaking and the addition of exogenous amylase are widely used modern techniques to alter the rate, extent and site of digestion (Yu, *et al.,* 1998; Zebeli *et al.*, 2010; Eastridge *et al*., 2010; Gibbens, 2014). Other techniques aim to decrease ruminal starch fermentation in an effort to reduce metabolic risks. Recently it has been shown that the treatment of wheat with a commercial starch binder effectively bound wheat starch in the rumen (Dunshea, 2012). Despite slower ruminal fermentation rates of maize compared to wheat, the impact of the starch binder on maize was not evaluated. The need therefore exits to determine the effect of the binder on various types of maize *in vitro.* As low vitreous maize ferments faster in the rumen (Ngonyamo-Majee *et al.,* 2008), specific interest will be on low vitreous maize

In vitro disappearance is the most common technique used to measure ruminal starch degradability in ruminants. With this technique ruminal starch disappearance can be measured directly (Menke *et al*., 1979; Huhtanen & Sveinbjörnsson, 2006). Indirect ruminal starch degradability can also be calculated by means of gas production techniques (Getachew *et al.*, 1998).

Vitreousness of grain refers to a higher content of hard endosperm in relation to soft endosperm in cereals (Larson *et al.,* 2008). The higher the hard endosperm content, the higher the vitreousness. Many documented studies indicate a reduction in *in vitro* and *in situ* starch degradability (Philippeau *et al.,* 2000; Correa *et al.,* 2002; Ngonyamo-Majee *et al.,* 2008; Larson & Hoffman, 2008) and decreased milk production (Taylor & Allen, 2005) with increased vitreousness.

1.6 Metabolic risks associated with high dietary starch consumption

High producing ruminant animals require large amounts of energy in the form of starch to optimize production efficiency. These high amounts of starch present in grain endosperm are needed without causing metabolic disorders such as SARA or acute acidosis (Nocek, 1997; Owens *et al*., 1998; Garrett *et al*., 1999). Increased rate of ruminal starch fermentation will result in a decrease in ruminal pH (Rowe *et al*., 1999). The risk of ruminal acidosis increases when ruminal pH decreases below 6 (Nocek, 1997). The ruminal rate and extent of starch fermentation are determined by and can

be summarized as follows:

- Vitreousness: The lower the grain vitreousness, the higher the ruminal starch fermentation rate (Philippe au *et al.,* 2000; Correa *et al.,* 2002; Ngonyamo-Majee *et al.,* 2008; Larson & Hoffman, 2008).
- Processing: Generally, the more processed, the higher the ruminal starch fermentation rate (Yu, *et al.,* 1998; Callison *et al*., 2001; Zebeli *et al.*, 2010; Eastridge *et al*., 2011; Gibbens, 2014). Starch binder treatment, in contrast, aims to decrease the rate and extent of highly fermentable starch in the rumen.
- Type of grain (wheat ferments faster in the rumen than maize) (Dunshea *et al*., 2012).

It could therefore be beneficial to shift some of the digestion of dietary starch from the fermentative areas to the small intestine in order to utilize the high dietary amounts of highly fermentable starch (as required to support high production) efficiently without metabolic risk.

1.7 Objectives

A study was conducted at the Stellenbosch University to determine the effect of vitreousness of maize and treatment of maize with a starch binder on milk production parameters of lactating dairy cows and the manipulation of starch digestibility. The aims of this study were to:

- Determine a rapid method to describe maize hardness via various techniques for application in the animal feed industry (NIR, PSI sieve, X-ray and RVA).
- Determine the potential practical use of predicting the rate of *in vitro* ruminal starch degradability by using NIR absorbance values.
- Investigate the possibility of changing ruminal starch disappearance characteristics with the treatment of maize of various vitreousness with a commercial starch binder, as measured by means of *in vitro* gas production *and in vitro* starch disappearance.
- Determine the effect of mill sieve size (1 mm vs 4 mm) and a starch binder treatment of low vitreous maize on *in vitro* starch disappearance.
- Investigate the possibility of changing apparent total tract starch digestibility and production data with the addition of a commercial starch binder on low vitreous maize *in vivo*.

1.8 References

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CHAPTER 2

Literature Review

2.1 Introduction

The history of maize began in 1492 when Christopher Columbus discovered this native North American grain in Cuba (Gibson *et al.,* 2002). As with all major grains, this was exported to Europe (Spain) presumably after Columbus's second visit to Cuba (Fox and Manley, 2009).

At first maize was only used as a garden crop in Europe, but soon it was recognized as a valuable food crop. It quickly spread to Italy, France and throughout Southwestern Europe and Northern Africa. By 1575 the crop had spread as far as China, the Philippines and the East Indies and was considered as a major food and feed crop.

Although maize is indigenous to the Western hemisphere, historians agree that it originated from the Tehuacan Valley in Mexico (Mangelsdorf, 1940). This could be established via the presence of maize pollen obtained from drill cores below Mexico City considered to be 80,000 years old. The original native form has nevertheless long been extinct.

Evidence suggests that cultivated maize arose through natural crossings with Gama grass (Tripsacum dactyloides) to yield teosinte (Galinat, 1984; Dickerson, 2003). Backcrossing of teosinte yielded primitive maize, which ultimately developed into the modern cultivars (Galinat, 1971).

Early North American expeditions indicated that maize had been grown extensively from southern North Dakota and both sides of the lower St. Lawrence Valley southward to Northern Argentina and Chile. It extended westward to the middle of Kansas and Nebraska, and an important lobe of the Mexican area extended northward to Arizona, New Mexico and Southern Colorado. It was also an important crop in the high valleys of the Andes in South America. Maize production took precedence over all activities for the Aztecs, Mayas, Incas and various Pueblo dwellers of the southwestern United States (Gibson *et al*, 2002).

Maize (*Maize mays L*) hardness (vitreousness) has long been recognized as an important quality characteristic that affects optimum rumen fermentation and ultimately animal production performance. Starch levels in modern dairy diets range between 25-30% of DM (Gencoglu *et al*., 2010). With TMR systems, this starch is mainly derived from the feeding of maize (Van Soest, 1994). It is generally accepted that maize is globally the most important and largest source of energy to lactating cows in order to meet ever increasing nutrient requirements. Vitreousness (hardness) of maize has a significant impact on digestibility and animal performance (Firkins *et al*., 2001).

Maize kernel hardness is principally a genetic expression, although the environment, maturity and post harvest handling also have an influence on hardness properties (Watson, 1987). Corona *et al*. (2006) further proposed that kernel vitreousness would also depend on the position on the ear as well as environmental conditions where it was grown. The phenomenon of maize vitreousness has been thoroughly studied to understand, predict and manage animal performance (Firkins *et al*., 2001; Philippeau *et al*., 1997; Correa *et al*., 2002; Allen *et al*., 2008; Ngonyamo-Majee *et al*., 2008ab; Lopes *et al*., 2009). An attempt to provide some insight on current literature of this phenomenon has been made. Maize morphology is discussed in depth. Furthermore a number of different techniques that are cited throughout the literature to predict vitreousness are discussed. These techniques are compared and evaluated as to their relevance to rumen kinetics and ruminant animal production potential. Various methods and techniques in literature to enhance and/or alter ruminal starch fermentation as well as total tract starch digestibility of maize of various vitreousnesses are also discussed.

2.2 Kernel Morphology

In the simplest of terms, maize consists of three main components: the pericarp, or outer protective covering, secondly the germ (embryo), and thirdly the endosperm (Kotarski *et al*., 1992). Figure 2.1 illustrates these basic components. Horny endosperm in Figure 2.1 refers to hard endosperm. Soft and hard endosperm is known as floury/opaque vs. horny/vitreous endosperm respectively. It is generally accepted that maize hardness is determined by the ratio of floury to vitreous endosperm (Watson, 1987; Paiva *et al.*, 1991; Delcour and Hoseney, 2010).

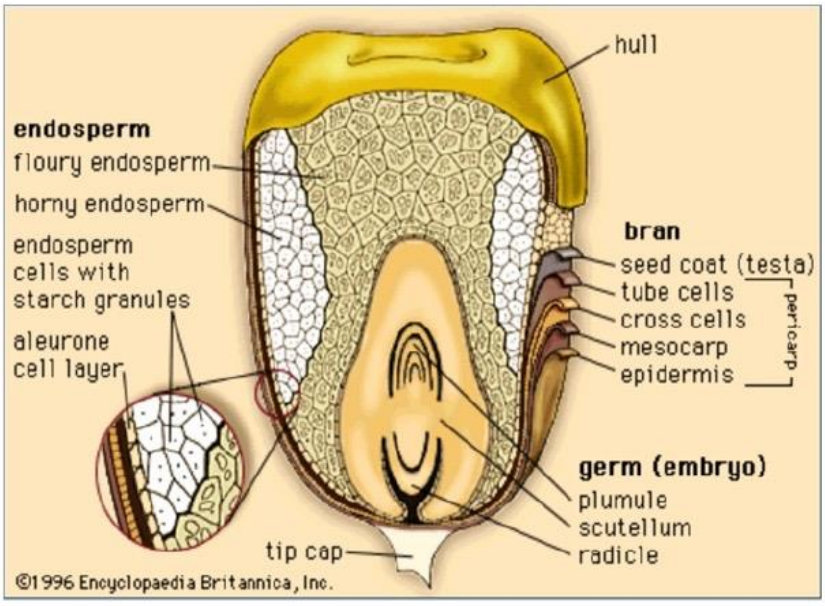


Figure 2.1 Maize kernel morphology (Encyclopedia Britannica, 1996).

2.2.1 Pericarp

The pericarp or seed coat is made up of various layers namely: tube cells, cross-cells, mesocarp and epidermis. In dry grain, the pericarp consists mostly of empty cells and serves to protect and support the growing endosperm and embryo (Evers *et al*., 1999; Owens and Zinn, 2005). In maize and sorghum the pericarp comprises about 5% to 6.5% of kernel weight (Wolf *et al.*, 1952, Rowe *et al*., 1999), whereas in oats it can be as much as 25%. The various components of the pericarp of maize are depicted in table 2.1. Almost 50% of the neutral detergent fibre (NDF) of the kernel is from the pericarp (Owens and Zinn, 2005). The NDF is the fraction that contains mostly cell wall constituents (cellulose, hemicelluloses and lignin) of low biological availability (Van der Merwe and Smith, 1991). Lower ruminal fermentation rates will result if the seed coat is hard and thick (Owens and Zinn, 2005). While starch ferments at a much faster rate than NDF in ruminants (Allen, 2007), the pericarp must however, be damaged, broken or cracked for rumen micro flora (RMO) to have access to endosperm to be able to ferment (Owens and Zinn, 2005).

2.2.2 Germ

Germ is made up of the scutellum, plumule and radicle and the combined embryonic axis (scutullum + plumule) make out approximately 12 to 15% of the kernel (Wolf *et al.*, 1952; Evers *et al*., 1999). See Table 2.1. Protein is present in far smaller quantities than starch in the maize kernel (Gibbon *et al*., 2003). Germ, in contrast to endosperm, does not contain any starch, but is rich in oil, protein, soluble sugars and some hormones (Serna-Saldivar, 2010). The scutellum, functions as a nutritive organ for the embryo (Watson, 1987), while the plumule will form the vegetative part of the plant (Evers *et al*., 1999). According to Owens and Zinn (2005), high-density maize hybrids further contains a larger portion germ with more oil. Owens and Zinn (2005) concluded that microbial yield would reduce due to substitution of starch by oil if these hybrids were fed to ruminants. According to Owens and Zinn (2005) microbial yield decreases because ruminants do not ferment oil as efficiently as a starch energy source.

Table 2.1. Proportions (%) of components of maize (Evers *et al*., 1999).

Maize Type	Hull	Pericarp + testa	Aleurone	Starchy Endosperm	Embryo Embryonic axis	Scutellum
Flint	-	6.5	2.2	79.6	1.1	10.6
Sweet	-	5.1	3.3	76.4	2	13.2
Dent	-	6		82	12	

2.2.3 Endosperm

It can be seen from Table 2.1 that endosperm is by far the greatest portion of a maize kernel and is about 80% (Kotarski *et al*., 1992; Opatpatanakit *et al*., 1994; Noziére and Michalet-Doreau, 1997; Evers *et al*., 1999) of the combined weight. Endosperm, as the largest tissue matter of grain, is constructed of two components i.e. soft and hard endosperm. This can be clearly distinguished from Figures 2.1 and 2.4. The starchy endosperm forms the majority of the seed and is packed with cells containing nutrients, which will be mobilized to support growth at the onset of germination (Evers *et al*., 1999). The endosperm cell nutrients are mainly carbohydrates in the form of starch (Giuberti *et al.*, 2014).

Starch and their granules have been the interest of researchers for hundreds of years. Van Leeuwenhoek (1719) used wheat starch as one of his subjects in his seminal work on microscopical discoveries. Figure 2.2 illustrates this groundbreaking work.

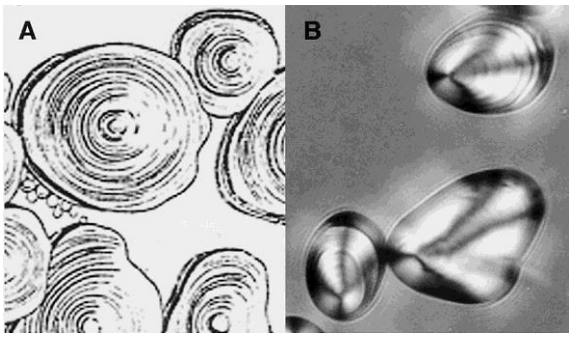


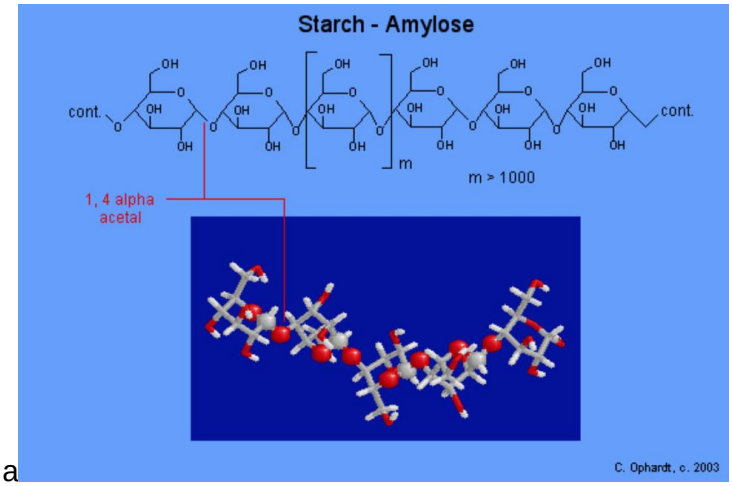
Figure 2.2. Starch granules. (A) An image of wheat grains as drawn by van Leeuwenhoek (1719) observed using the first microscope. (B) A modern image of potato starch granules viewed under polarized light (Wang *et al*., 1998).

2.3 Types of starch

In maize, starch is chemically present in primarily a branched chain polymer named amylopectin and a smaller amount of the linear polymer amylose (Wang *et al*., 1998; Rowe *et al*., 1999; Huntington *et al*., 2006). Amylopectin has a less crystalline structure and a higher solubility, and is more rapidly broken down by amylase than the more linear amylose (Rowe *et al*., 1999). A lower temperature is further required for gelatinising starches containing lower levels of amylose (Rowe *et al*., 1999).

Figure 2.3 indicates the differences between amylose and amylopectin. Figure 2.3a shows a very small portion of an amylose chain, which normally consists of long polymer chains of glucose units connected by a α-acetal linkages.

a



b

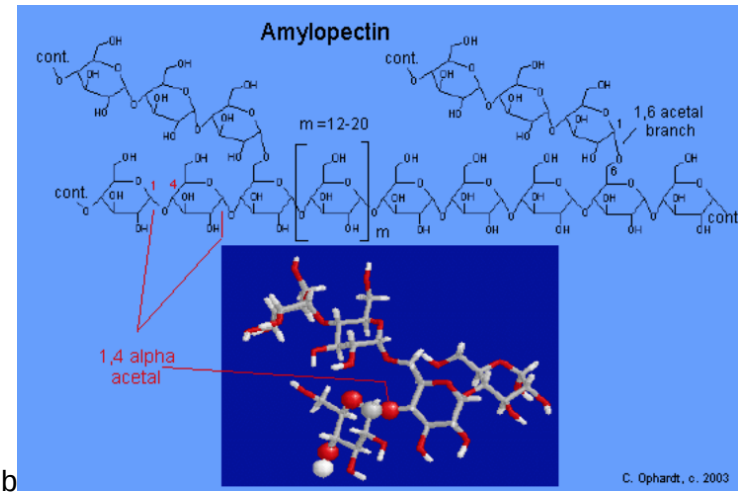


Figure 2.3. Chemical structure of starch. a = Amylose, b = Amylopectin (Ophardt, 2003).

Figure 2.3b in contrast shows a very small portion of an amylopectin type structure showing two branch points. The acetal linkages are α-connecting C #1 of one glucose to C #4 of the next glucose (Ophardt, 2003). Amylose, with a strong linear structure, is less fermentable than the multi-branched structure of amylopectin (Huntington *et al*., 2006). Corona *et al*. (2006) suggests that the tighter intermolecular bindings between amylose starch molecules render amylose starch less fermentable than amylopectin and thus more resistant to ruminal fermentation. Although the amylose content can be as low as 2%, typically in maize it is between 24 and 30% (Owens and Zinn, 2005). Starches from most cereal grain species are composed of about 30% amylose and

70% amylopectin (Wang *et al*., 1998). Waxy (softer) genotypes of maize generally have higher levels of amylopectin in the endosperm (almost 100%) and non waxy (harder) varieties have less amylopectin (75%) and more amylose (25%) (Rooney and Pflugfelder, 1986). Dickerson (2003) in agreement suggests that waxy genotypes carry a gene that produces almost 100% amylopectin. Generally, the higher the amylose content, the higher the vitreousness and the higher the ruminal resistance of the starch (RRS) and therefor the lower the ruminal fermentation.

2.4 Maize Classification

Maize is classified as flint, popcorn, dent, flour and sweet according to the physical shape of the kernels, the pattern of endosperm composition as well as quantity and quality of endosperm (Dickerson, 2003; Corona *et al*., 2006; Fox and Manley, 2009). See Figure 2.4. Although flint (*Zea indurate*) and dent (*Zea indentata*) kernels are both intermediate with respect to hardness, Wolf *et al*. (1952) reported a ratio of 2:1 of vitreous to floury endosperm. Dent maize kernels are generally softer than flint kernels (Dickerson, 2003). The endosperm in floury (*Zea amylacea*) kernels is almost all soft (Corona *et al*., 2006). Pop kernels (*Zea everta*) are round and short with a very large portion of vitreous endosperm (Fox and Manley, 2009), whereas the wrinkled, glassy appearance of sweet maize kernels is the result of a sugary gene that retards the normal conversion of sugar to starch during endosperm development (Dickerson, 2003). Figure 2.4 shows the endosperm distribution of the various maize types.

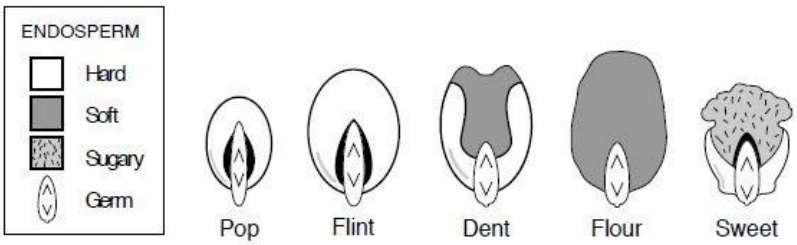


Figure 2.4. Endosperm distribution of types of maize (Dickerson, 2003).

2.5 Maize storage protein

Storage protein in grains is known as prolamin (Larson and Hoffman, 2008) and is named so after the high *prol*ine and glut*amine* contents found in these proteins (Fox and Manley, 2009). The proline in a specific cereal has been given specific names to easily identify the specific storage protein families (Fox and Manley, 2009; Hoffman and Shaver, 2009):

- wheat (gliadin)
- barley (hordein)
- rye (secalin)
- maize (zein)
- sorghum (kafirin)
- oats (avenin)

Although modified endosperm types exist in maize that are low in prolamins, the cereal grains (wheat, oats, barley) have a lower prolamin content than maize (Corona *et al*., 2006).

Proline is a highly hydrophobic amino acid capable of complex folding and thus proteins with high proline contents develop tertiary structures that are intensely hydrophobic and are only soluble in aqueous alcohol solutions (Momany, *et al.,* 2006). Zein is therefore insoluble in water and ruminal fluid (Rowe *et al*., 1999). Paulis and Wall (1977) also reported that the glutelins can be extracted by alkali and that the kafirins are soluble in alcohol. This suggests that chemical treatment using alkali and/or alcohol may be useful in modifying the endosperm and improving starch digestibility of sorghum. Further evidence that the protein content of the endosperm is a primary factor limiting starch digestion is the finding of increased glucose release following pre-treatment of sorghum endosperm with the proteases, “Pronase” or pepsin (Kotarski *et al*., 1992).

Four types of zein have been identified: alpha (α), beta (β), gamma (γ), and delta (δ) (Lending and Larkins, 1989). Maize hardness is thus also dependent on the ratio of zein rich protein in relation to starch. Zein bodies adhere to maize starch granules and form an extremely strong starch protein matrix (Lee *et al*., 2006; Hoffman and Shaver, 2009) to create vitreous maize. According to Abdelrahram and Hoseney (1984) chemical bonding of the protein, rather than physical attachment to starch granules are responsible for this strong matrix. Chandrashekar and Mazhar (1999) suggest that amorphous, non-crystalline amylopectin molecules at the surface of starch granules in

maize endosperm can interact and form contacts that link starch granules together. These contacts could provide a mechanism that complements the one postulated for γ-zein rich protein bodies, which are proposed to fill the spaces between starch granules and crosslink proteins, creating a vitreous kernel phenotype. Figure 2.5 shows this strong zein protein matrix in hard maize compared to the more loosely packed starch of soft maize.

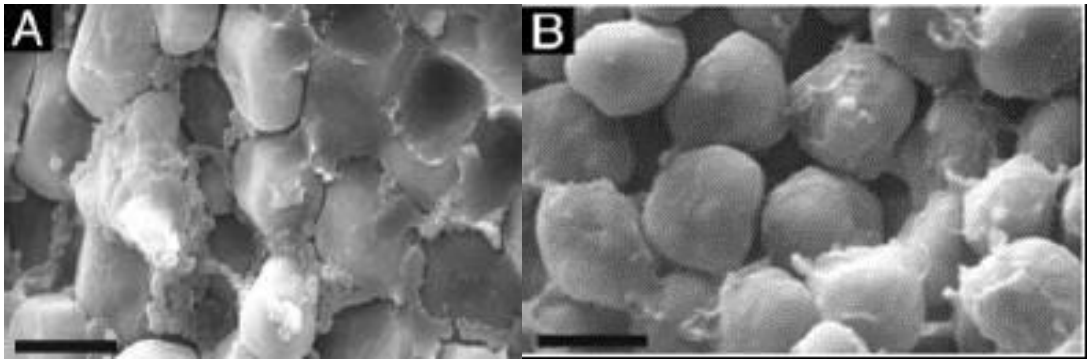


Figure 2.5. Scanning electron microscopy of starch granules in maize: A) starch granules strongly imbedded in a zein-protein matrix, B) starch granules in floury maize endosperm with less extensive encapsulation by zein (Gibbon *et. al*., 2003).

It is thus apparent that maize kernels will develop with a specific ratio of vitreous to floury (V:F) endosperm depending on stage of maturity and the intrinsic genetic code of the particular hybrid of maize (Watson, 1987; Erasmus, 2003). While the vitreous endosperm is extremely hard, the floury endosperm is full of void spaces or micro fissures (Philippeau *et al*., 1999). Genotypic flinty kernels will have a higher V:F than floury types (Owens, 2005). Underlying DNA (Gibbon *et. al*., 2003), environmental conditions (Opatpatanakit *et al*., 1994) as well as stage of maturity (Philippeau *et al*., 1997) will all impact on the V:F endosperm ratio. It is therefor generally agreed that the relation of V:F endosperm determines maize kernel hardness and that this specific ratio is determined by the presence of zein (Watson, 1987; Paiva *et al.*, 1991; Dombrink-Kurtzman and Bietz, 1993; Eyherabide *et al.*, 1996; Robutti *et al.*, 1997; Chandrashekar and Mazhar, 1999; Lee *et al.*, 2006; Delcour and Hoseney, 2010). It has been well documented that vitreousness of maize has a significant impact on rumen function and fermentation, as well as on total tract starch digestibility (Ngonyamo-Majee *et al*., 2008ab).

2.6 Starch digestion

The impact of kernel endosperm vitreousness on ruminal fermentation has been well documented. In some instances it would be beneficial to increase ruminal starch fermentation (Philippeau *et al*., 1999; Szazs *et al*., 2007; Allen *et al*., 2008; Ngonyamo-Majee *et al*., 2008ab), while decreasing ruminal starch fermentation will be especially of importance where very high amounts of highly fermentable starch are fed in an effort to decrease the risk of metabolic disorders (Nocek, 1997; Owens *et al*., 1998; Garrett *et al*., 1999).

A number of studies have shown that ruminal starch degradability is strongly and negatively correlated with endosperm vitreousness (Philippeau *et al*., 1997; Correa *et al*., 2002; Allen *et al*., 2008; Lopes *et al*., 2009). Ngonyamo-Majee *et al*. (2008b) showed the negative linear relationship *in situ* (Figure 2.6).

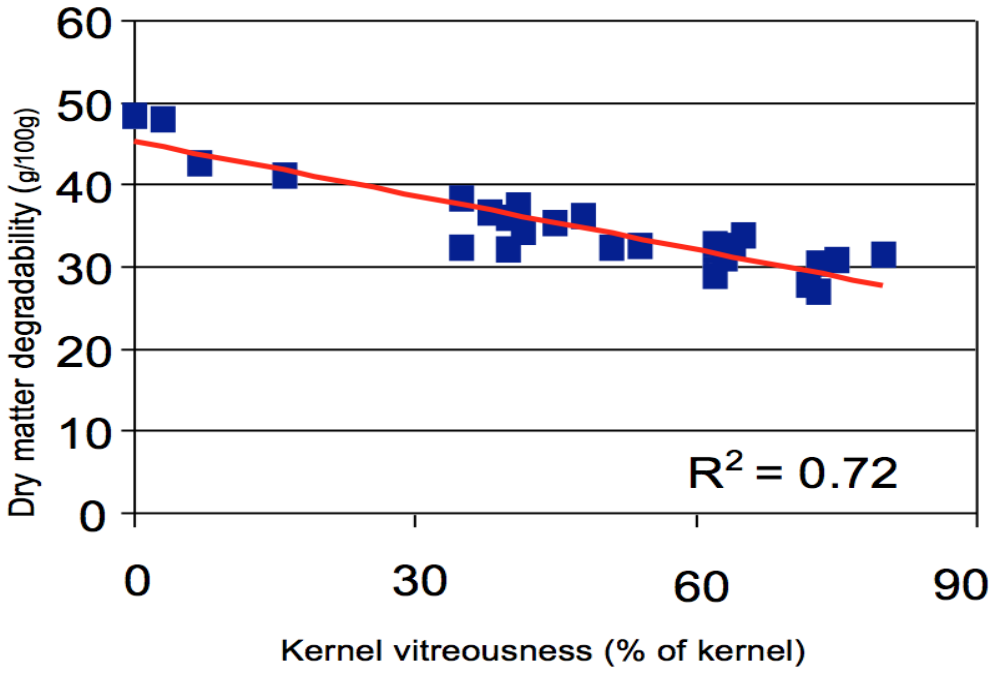


Figure 2.6. The effect of maize vitreousness on ruminal starch degradability (Ngonyamo-Majee *et al*., 2008b).

Ruminal degradation and fermentation will be limited when high vitreous maize is fed due to maize starch granules which are surrounded by zein and thus being encapsulated in a tight protein (zein) starch matrix (Kotarski *et al*., 1992; Johnson *et*

al., 1999; Gibbon *et al*., 2003). This strong starch protein matrix, limit rumen micro-organisms (RMO) access to kernel starch and are responsible for slower ruminal starch fermentation rates than with other cereal grain (Rooney and Pflugfelder, 1986; McAllister *et al*., 1993; Opatpatanaki *et al*., 1994).

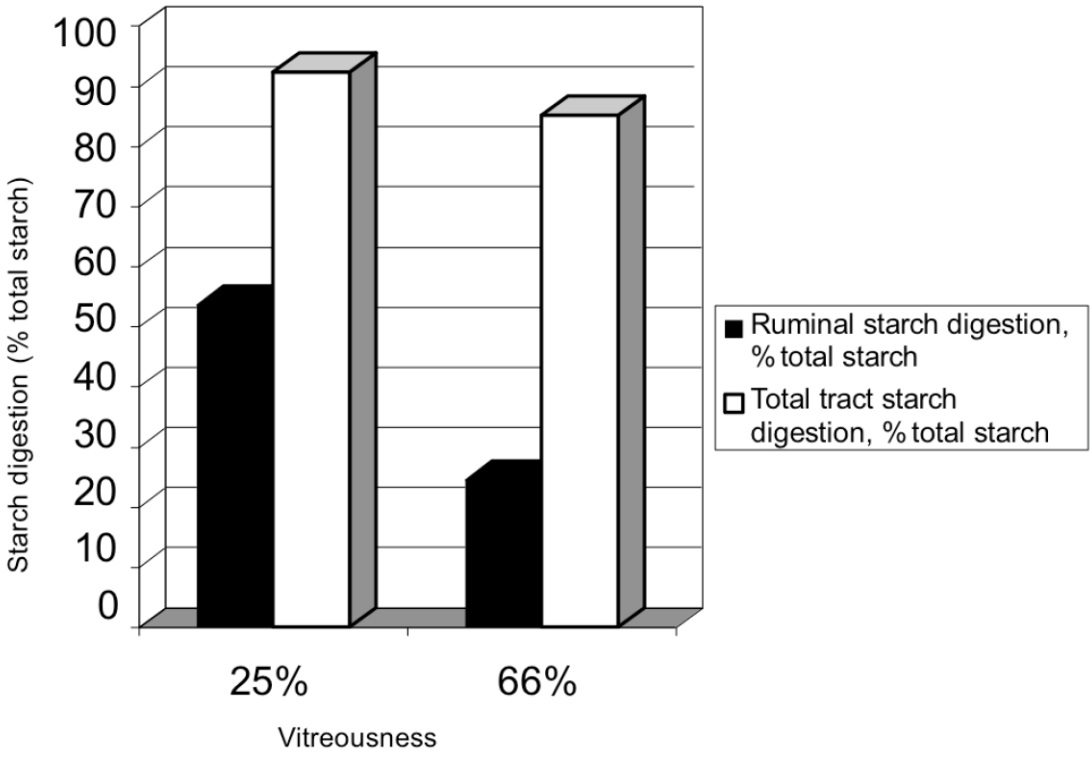


Figure 2.7. The effect of maize vitreousness on total tract and ruminal starch degradation (Allen *et al*., 2008).

Based on a survey of 14 common maize types Philippeau *et al*. (1999) concluded that 85% of ruminal (*in situ*) starch disappearance could be attributed to vitreousness. Both ruminal and total tract starch digestibility values were shown (Figure 2.7) by Allen *et al*. (2008) to decrease as vitreousness of maize increased. This is in accordance with work done by Ngonyamo-Majee *et al*. (2008b) as indicated in Figure 2.6. Correa *et al*. (2002) further also showed that kernel endosperm vitreousness increased with advancing maturity and decreased ruminal *in situ* starch disappearance. By comparing maize hybrids at two maturities Philippeau *et al*. (1997) found that vitreousness differed between dent and flint maize genotypes (26.5 vs. 38.3%), but vitreousness differed more between immature and mature grains (32 vs. 60.2%). Philippeau *et al*. (1997) further reported a high correlation coefficient (r^2 = 0.93) between an increase in

vitreousness and maturity of maize. Earlier Murphy and Dalby (1971) reported the impact of maturity on vitreousness of maize as shown in Figure 2.8. It was shown by Murphy & Dalby (1971) that the zein content increase with maturity in all maize genotypes, except floury genotypes (Figure 2.8).

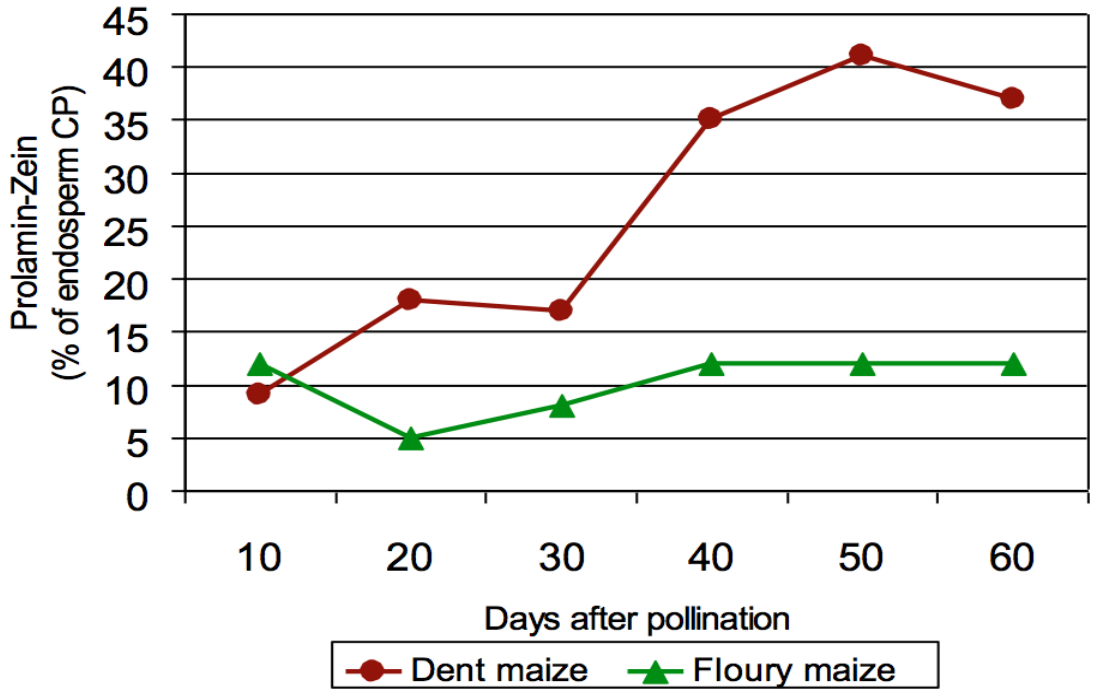


Figure 2.8. The effect of maize kernel maturity on zein contentment (Murphy and Dalby, 1971).

These findings are in accordance with the work of McAllister *et al*. (1990) as depicted in Table 2.2. While crude protein (CP) composition essentially remained the same, both starch and vitreousness content increased with stage of maturity irrespective of genotype (McAllister *et al.,* 1990).

Table 2.2. Influence of genotype and stage of maturity on the chemical composition (%DM) and physical parameters of grain (McAllister *et al*., 1990).

Genotype	Days after silking	DM Content		Chemical Composition		Physical Parameter
		Whole plant	Grain	CP	Starch	Vitreousness
Dent	22	29.0	38.7	12.5	61.0	26.5
	37	36.5	56.0	10.1	63.1	28.0
	59	39.8	66.0	11.1	68.5	45.4
	78	50.7	57.7	10.0	68.6	48.1
Flint	22	24.7	36.9	13.3	58.6	38.3
	34	30.2	53.9	11.4	62.1	53.3
	48	35.9	63.3	10.4	67.9	61.5
	65	39.3	70.5	11.6	67.3	66.2
	78	40.1	75.1	11.3	67.2	72.3

In a study with feedlot steers to determine the influence of moisture on vitreousness and ruminal starch disappearance, Szazs *et al*. (2007), reported both lower ruminal and total tract starch disappearance as vitreousness increased. Huntington (1997) further concluded that by feeding waxy genotypes of maize or sorghum, animal performance increase even with dry processing (cracking, rolling), compared to the flinty genotypes, indicating more complete digestion of starch with a lower amylose content.

Higher vitreousness could therefore be exploited to decrease the rate and extent of ruminal starch fermentation. Decreased total tract starch digestibility is, albeit to a lesser degree (see Figure 2.7), comparable to decreased ruminal starch fermentation, also associated with high vitreousness (Firkins *et al*., 2001; Szazs *et al*., 2007; Ngonyamo-Majee *et al*., 2008b; Allen *et al*., 2008). This phenomenon often leads to decreased animal performance with high vitreous maize (Huntington, 1997), but does not manifest similarly in various farm animal species.

2.6.1 Specie differences

In a comprehensive overview Rowe *et al.* (1999) summarizes some major differences among animal species in efficiency of intestinal carbohydrate digestion, and these are summarized in Table 2.3. Maize has the highest apparent digestibility in poultry, but is very poorly digested in the small intestine of the horse, even when it is finely ground.

Furthermore sheep seems to digest maize starch better both ruminaly, as well as in the lower intestine, resulting in significant higher starch utilization by sheep than by cattle (Rowe *et al*., 1999).

Table 2.3. Differences among livestock species in their ability to digest different cereal grains (Rowe *et al*., 1999).

	Maize	Sorghum	Barley	Wheat	Oats
		Total tract digestibility (% of starch intake)			
Cattle	93	87	93	98	98
Sheep	100	97	100		
Pigs		100	99	100	
Poultry	100	99	100	100	100
		Small intestine (pre-ileal) (% of starch entering stomach)			
Cattle	66	63	73	85	76
Sheep	96	71	73		
Pigs		72-94	93	98	
Poultry	85	85	80	82	
Horses	30	35	25		85
		Fermented in rumen (% of intake)			
Cattle	76	64	87	89	92
Sheep	86	85	94		

Nocek and Tamminga (1991) indicated that rumen degradable starch as percentage of total starch of whole maize varies from 58.9 to 75.0% when fed to cattle and sheep, respectively. With ground maize, the values varied from 71.4 to 93.0% in cattle and sheep, respectively. It was shown that total tract starch digestibility was almost 100% (Table 2.3) when sheep were fed rolled barley (MacRae and Armstrong, 1969). In contrast, between 18 and 35% maize can pass undigested through the total tract of cattle (Morrison, 1959).

The mechanics of this higher starch digestibility of sheep vs. cattle is likely to be related to differences in digestive capacity and the different sizes of sheep and cattle intestinal tracts (see Figure 2.9). The dynamics of particle flow through the tract (Rowe *et al*., 1999) and the ability of sheep to chew the grain into smaller particles (Van Soest, 1994) also play a role. Despite the specie differences, it is clear that starch digestion in the total digestive tract of ruminants generally exceeds 95% (Tucker *et al*., 1968).

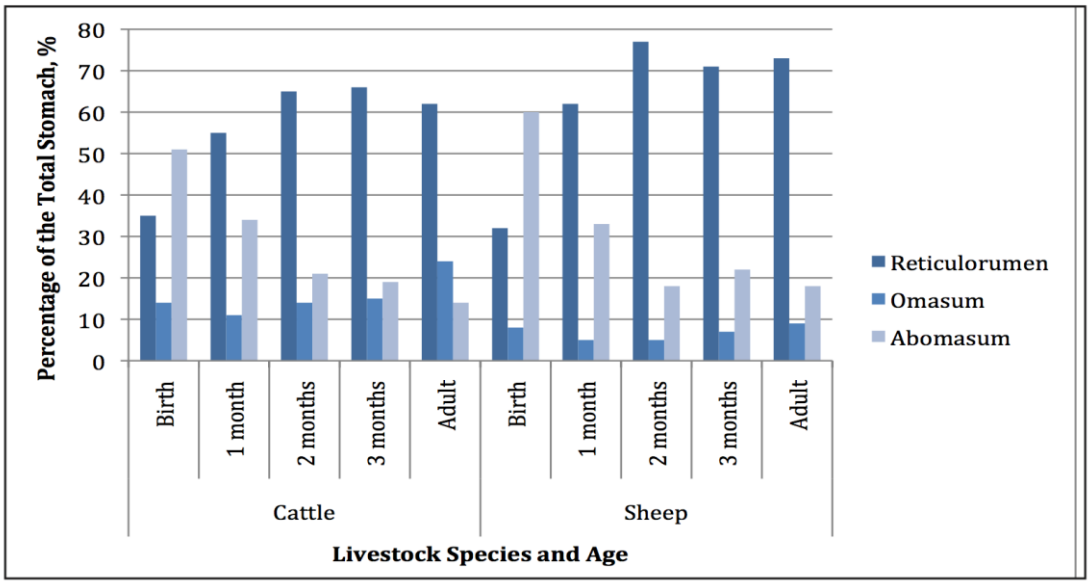


Figure 2.9. Relative proportions of stomach compartments in cattle and sheep at various ages (Parish *et al*., 2009).

The available energy of grains to various species (including non ruminants) cannot be directly related to starch content because of changes due to conditions like stage of maturity, type and composition of endosperm, environmental influences and underlying genetic code (Black, 2008). Figure 2.10 indicates that the available energy of grains is not always directly related to the starch content (Black, 2008). This suggests that energy required for animal maintenance and production cannot be measured as a mere function of ME (Black, 2008).

Ruminants digest starch at various degrees at three intestinal sites:

- Rumen
- Small intestine
- Lower intestine

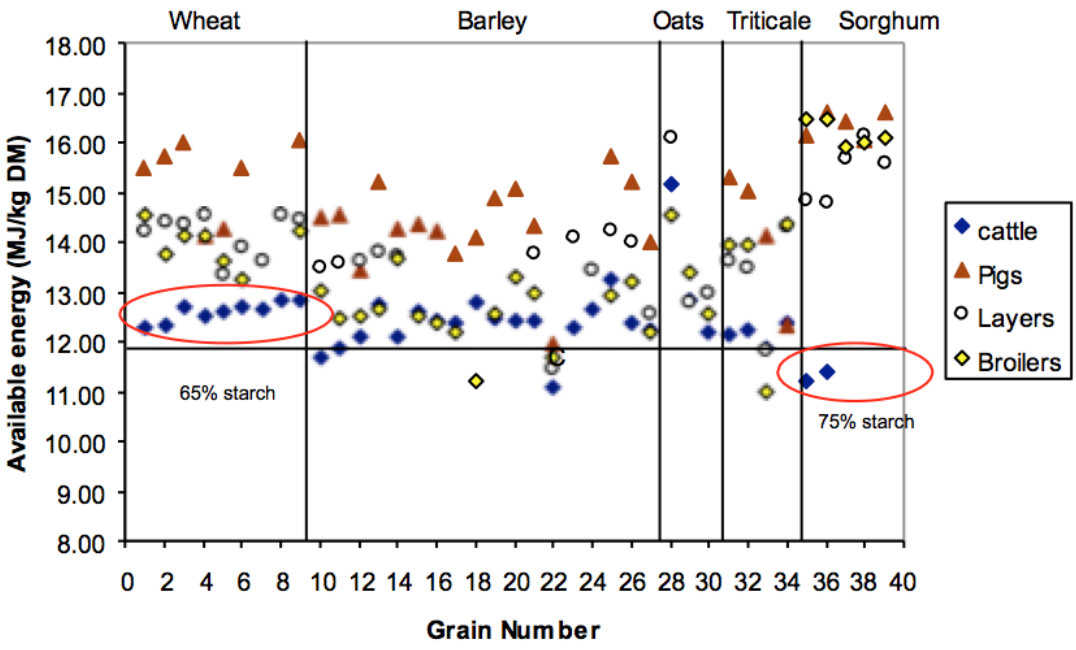


Figure 2.10. Available energy content of individual grain samples fed to animals *ad libitum.* Values for pigs are DE, for poultry AME and for cattle ME (Black 2008).

2.6.2 Ruminant starch digestion pathways

Starch digestion pathways in the ruminant are an intricate combination of ruminal fermentation, small intestinal digestion and large intestinal fermentation. Despite intricate knowledge, the complexity of the process is still not clearly understood as the extent and site of digestion and absorption of starch is dependent on many variables such as species, diet, grain type, processing method and the extent of grain processing.

Figure 2.11 is a schematic presentation of the various starch digestion pathways in the ruminant. The rumen resistant starch (RRS) is not digested in the rumen, which results in low concentrations of short-chain fatty acids (VFA) and a higher ruminal pH. Non rumen resistant starch (NRS) is degraded in the rumen and leads to a release of VFA, changing the proportions of acetate (C_2): propionate (C_3) and butyrate (C_4), as well as decreasing the ruminal pH (high risk of rumen acidosis). The undigested RRS is mostly degraded in the small intestine by pancreatic amylases, while some portions of it can be degraded in the large intestine (hind gut fermentation). The model also indicates the mechanisms of removal of VFA (metabolism of VFA to beta-hydroxybutyrate

(BHB), acetoacetate (AcAc), and lactate from the rumen and the absorption of glucose from the small intestine.

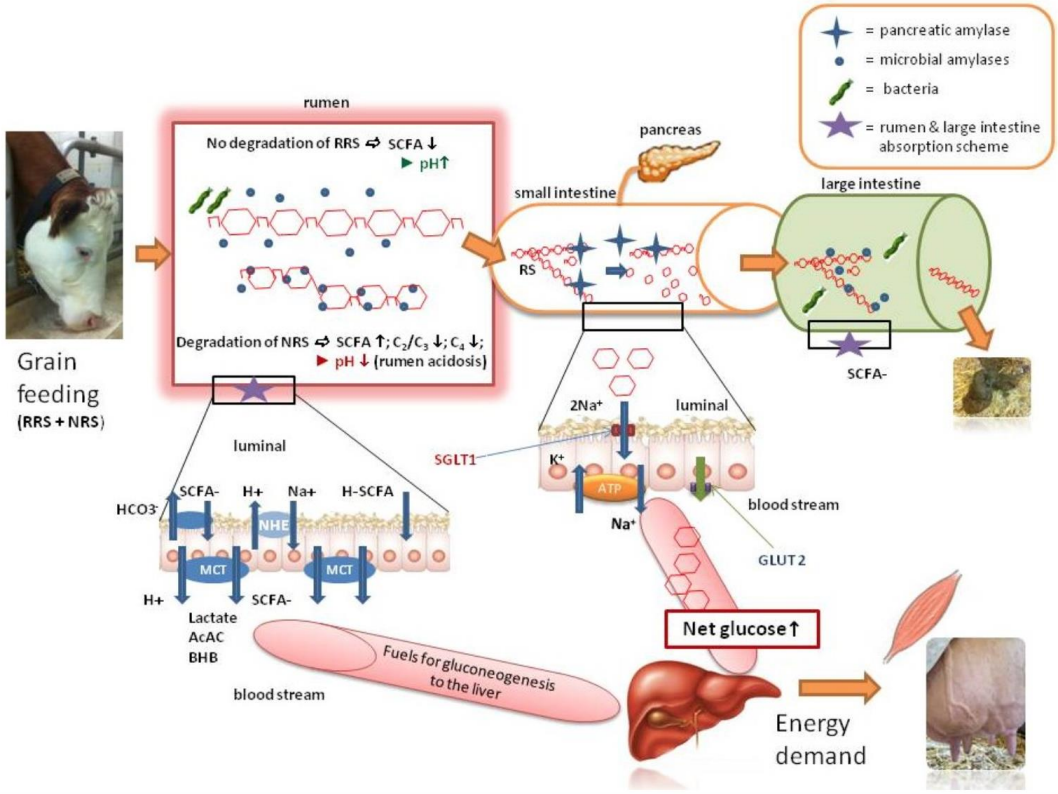


Figure 2.11. A simplified model describing the fates of ruminally resistant starch (RRS) and non-RRS (NRS) fed to cattle (Deckhardt *et al*., 2013).

2.6.2.1 Ruminal fermentation

The starch that is fermented in the rumen is referred to as non ruminant resistant starch (NRS) (Deckhardt *et al*., 2013). Specific amolytic RMO's are responsible for the fermentation of NRS. Starch degraded in the rumen leads to a release of VFA's; mainly decreasing the ratio of acetate (C_2) to propionate (C_3) proportions (Chen *et al*., 1995; Fredin *et al.,* 2015). When investigating the effects of rapidly degradable starch on ruminal degradation in dairy cows, Lechartier and Peyraud (2011) reported a decrease in acetate (C_2) to propionate (C_3) ratio from 2.7 to 2.1 when the intake of highly fermentable starch was increased. *Ruminobacter amylophilus* and *Streptococcus bovis* are the most prominent ruminal starch fermenting bacteria compared to ruminal

fungi and protozoa which are to a lesser extend involved (Huntington, 1997). *Prevotella ruminicola* and some *Butyrivibrio fibrisolvens* strains were also shown to significantly contribute to ruminal starch fermentation (Tricarico *et al*., 2005). Starch particles are attacked by α-amylase enzymes produced by microbes and are digested from the outer surface to the inside (Cone, 1991; Huhtanen & Sveinbjörnsson, 2006; Gibbens, 2014). *Bacteroides amylophilus, Bacteroides ruminocola, Butyrivibrio fibrisolvens, Selenomona lactylitica, Prevotella ruminocola, Streptococcus bovis, Eubacterium ruminantium, Ruminococcus bromii, Ruminobacter amylophilus, Succinimonas amylolytica* and *Lactobacillus spp*. are some of the RMO's shown to be responsible for the production of the various ruminal amylolytic (mostly α-amylase) enzymes (Kotarski *et al*., 1992). The two primary enzymes that hydrolyze starches are alpha (α)- and beta (β)-amylase (Van Soest, 1994). While α-amylase cleaves both amylose and amylopectin, β-amylase cleaves units from the ends of chains (Van Soest, 1994). Nevertheless, β-amylase activity is limited to the peripheral parts of amylopectin (Van Soest, 1994). The enzymes α-amylase, β-amylase, R-enzyme, pullulanase, iso-amylase is all produced by the RMO's (Cerrilla & Martínez, 2003).

The extent and rate of ruminal starch fermentation is dependent on variables such as species, diet, grain type, processing method and the extent of processing. Increased ruminal starch fermentation will result in a lowering of pH (Rowe *et al*., 1999) and an increased propionate to acetate ratio (Chen *et al*., 1994; Deckhardt *et al*., 2013).

2.6.2.2 Small intestinal digestion

With low starch diets, intestinal starch digestion is of little importance due to small quantities of alpha-linked glucose polymers that pass to the abomasum (Heald, 1951). In contrast, in starch rich diets; and depending on the type of the grain, the extent of processing prior to feeding, and the species of animal fed, an appreciable amount of starch and protozoal glycogen may escape fermentation in the rumen and enter the small intestine (Cerrilla and Martínez, 2003). Whole grain, with an intact pericarp, is almost completely resistant to ruminal fermentation because microbes are unable to attach to the whole kernels (Callison *et al*., 2001; Eastridge *et al*., 2010). Therefore, the higher the amount of rumen resistant starch (RRS) fed, the higher the amount of starch present in the abomasum and duodenum. The abomasum produces hydrochloric acid (reducing pH to 2.5) and digestive enzymes, such as pepsin, while also receiving digestive enzymes secreted from the pancreas, such as pancreatic

lipase and amylase (Constable *et al.,* 2006). Both α and β-amylase are secreted by the pancreas and are responsible for most of the starch hydrolysis in the abomasum and duodenum. Maltotriose (end product of amylase starch hydrolysis of amylose and amylopectin) consists of four to eight glucose moieties and may still contain the alpha-(1-6) linkage(s) that cannot be hydrolyzed by amylases. According to Clark and Bauchop (1977) as cited by Cerrilla and Martínez (2003), debranching enzymes (R-enzyme, pullulanase. iso-amylase, or alpha-limit dextrinase) are required to break these bonds. The small intestine follows the abomasum as a further site of nutrient absorption. Digesta entering the small intestine mix with enzymatic secretions from the pancreas and liver, which elevate the pH from 2.5 (abomasum) to between 7 and 8 (Cerrilla and Martínez, 2003). This higher pH is needed for enzymes in the small intestine to function efficiently. Bile from the gall bladder is secreted into the first section of the small intestine (duodenum) to aid in digestion. Active nutrient absorption occurs throughout the small intestine (Deckhardt *et al*., 2013).

The capacity of the ruminant small intestine to digest large amounts of starch has nevertheless been questioned (Waldo, 1973; Croome *et al*., 1992), due to:

- Relative low levels of pancreatic amylase, such as intestinal maltase and isomaltase (Siddons, 1968; Coombe and Siddons, 1973; Coombe and Smith, 1974).
- Relative low glucose absorption capacity (Ørskov, 1986; Kreikemeier *et al*., 1991; Tanigushi *et al*., 1995).

However, in contrast it has also been suggested that starch digested post ruminally is used more efficiently than that digested in the rumen (Nocek and Tamminga, 1991). Ruminant animals may be capable of digesting large amounts of starch in the small intestine through an adaptation in the activity of the host carbohydrases (Janes *et al*., 1985).

Both decreased amylase secretion (Swanson *et al*., 2002) and enzyme activity (Kreikemeier *et al*., 1990) have been found with the presence of glucose or starch hydrolysate in the bovine small intestine. Gastrointestinal hormones might thus regulate pancreatic enzyme secretion. Kreikemeier *et al*. (1990) reported a higher amylolytic activity when a high protein lucerne hay diet was fed vs. a grain diet, with equal amounts of energy. This could be related to the stimulation of the pancreas by the protease sensitive cholecystokinin releasing peptide due to the presence of protein in the intestine (Fushiki *et al*. 1989). It is thus possible that pancreatic secretion in

ruminants might be mediated by a monitor peptide (Fushiki *et al*. 1989). Results from Kreikemeier *et al*. (1990) also suggest that the amount of protein in the diet could play an important role in starch digestion in the small intestine.

Despite inconsistency in the literature as to the effectiveness of intestinal starch digestion in ruminants, intestinal starch digestion in ruminants is in essence similar to that in monogastic animals (Cerrilla and Martínez, 2003).

6.2.2.3 Large intestinal fermentation

Microbial fermentation of carbohydrates in the hindgut of dairy cattle is responsible for 5 to 10% of total tract carbohydrate digestion (Gressley *et al.,* 2011). Carbohydrates are fermented to VFA and gas at similar rates in the hindgut as in the rumen (Hume, 1997; Váradyová *et al.,* 2000). Volatile fatty acid profiles in both locations respond similarly to changes in substrates while the majority (>95%) of VFA produced in the hindgut are passively absorbed across the intestinal epithelium (Argenzio *et al.,* 1975; Engelhardt and Rechkemmer, 1983).

When dietary, animal, or environmental factors contribute to abnormal, excessive flow of fermentable carbohydrates from the small intestine, hindgut acidosis can occur (McCarthy *et al*., 1989; Godfrey *et al*., 1993; Overton *et al*., 1995; Shabi *et al*., 1999). Hindgut acidosis is characterized by increased rates of production of short-chain fatty acids including lactic acid, decreased digesta pH, and damage to gut epithelium as evidenced by the appearance of mucin casts in feces (Gressley *et al.,* 2011). Conditions such as sub-acute rumen acidosis (SARA) that increase post ruminal flow of fermentable carbohydrates may cause increased hindgut fermentation (Hall, 2002; Lazier *et al.,* 2008). Hindgut acidosis is thus more likely to occur in high producing animals fed diets with relatively greater proportions of grains and lesser proportions of forage. An inflammatory response results in a breach of the barrier between animal and digesta and may lead to laminitis.

2.7 Site of starch digestion

The method of grain processing affects the site of digestion of starch in ruminants. Wu *et al*. (1994), found in cows fed steam flaked sorghum that the main site of starch

digestion was the rumen. In cows fed dry rolled sorghum, starch was mainly digested in the lower intestine. Table 2.4 indicates the major advantages and disadvantages of site of starch digestion as summarized by Rowe *et al.* (1999).

Table 2.4. Significance of site of digestion in determining nutritional value of grain (Rowe *et al*., 1999).

Positive features	Negative features
Rumen fermentation	
Microbial protein and vitamins available for intestinal absorption	Acid accumulation and low pH leads to: risk of acidosis, reduced fibre digestion
VFA absorption provides metabolisable energy	Energy loss through heat, CH_4, and H_2
Intestinal digestion	
No fermentation energy losses	No microbial protein production
Glucose absorbed which can increase marbling	
Hindgut fermentation	
VFA absorption provides metabolisable energy	Acid accumulation and low pH leads to: risk of acidosis, reduced fibre digestion
	Energy loss through heat, CH_4, and H_2

According to these authors, it is beneficial to the animal to maximize the digestion of starch and absorption of glucose from the small intestine. This is based on the energetic efficiency of intestinal digestion being approximately 30% higher than fermentative digestion (Nocek and Tamminga, 1991). Increased amounts of starch could also escape ruminal degradation by increased rumen fluid dilution rate (Cerrilla and Martínez, 2003). The dilution rate of rumen fluid is higher with long roughages than with ground roughages (Hodgeson and Thomas, 1975) and is related to the greater amount of time spent ruminating. In a study with lambs fed different lengths of roughage, the amount of ground maize starch that passed to the duodenum of sheep doubled when ground straw was replaced with long straw (Thompson and Lamming, 1972; Thompson, 1973). Ørskov *et al*. (1969) earlier reported similar results. Intestinal starch digestion also carries no risk of acidosis as with ruminal fermentative starch digestion. Bovine amylase appears to be pH sensitive as ruminal starch fermentation

has been shown to improve by the addition of buffers (Wheeler *et al*., 1977). This negative effect of high dietary starch is related to more rapid fermentation and the development of large amounts of lactic acid as primary product and a subsequent lower sub optimal ruminal pH (Van Soest, 1994). In contrast, Theurer *et al*. (1999) showed that starch supplementation to the rumen is more beneficial to milk yield than compared to post rumen intestinal supplementation of starch.

In general, to date results indicate post ruminal bypass starch utilization is inferior to that of bypass protein (Van Soest, 1994). Despite variable current results, it appears to be and would be extremely beneficial to ruminants to shift some starch digestion from fermentative areas to the small intestine. This is due to the lower risk of SARA or acute acidosis.

2.8 Sub acute rumen acidosis (SARA) and acute clinical acidosis

Dairy cattle consume large amounts of starch (20-40% of diet DM) as a way to increase energy consumption (ME) in support of high milk production (Patton *et al*., 2011). These high amounts of starch present in endosperm are needed without causing metabolic disorders such as acute acidosis or SARA (Nocek, 1997; Owens *et al*., 1998; Garrett *et al*., 1999).

Acute acidosis and SARA are defined as occurring when ruminal pH is reduced below 5.0 and 5.6, respectively (Krause and Oetzel, 2006; Penner *et al.,* 2007; Radostits *et al.,* 2007).

Under normal operating conditions, the interior of the bovine rumen has a pH of 6.5 to 7.2 (Nocek, 1997; Van Winden *et al*., 2002). In contrast, the lower intestine (abomasum) of the ruminant is more acidic with a pH of 2 to 3 (Geishauser *et al.,* 1996; Van Winden *et al.,* 2002; Constable *et al.,* 2006).

Increasing starch fermentation in the rumen increases propionic acid as a proportion of total VFA in the rumen (Chen *et al*., 1994). Propionic acid is a major gluconeogenic precursor in ruminants, and increasing the proportion of propionic acid might result in:

- A higher net energy absorption from the rumen
- An increase in glucose synthesis by the liver
- A reduction in the use of AA for milk protein synthesis (Theurer, 1986)
- Ultimately improved animal performance.

Unfortunately an increased rate of ruminal starch fermentation will almost always result in a decrease in ruminal pH (Rowe *et al*., 1999). The risk of ruminal acidosis increases when ruminal pH decreases below 6 (Nocek, 1997). The use of highly fermentable starch, such as wheat, will also decrease fibre digestion (Leddin *et al.,* 2009) because of a lower ruminal pH. In a study with lactating dairy cows fed different levels of crushed wheat, Leddin *et al*. (2009) reported that neutral detergent fibre (NDF) digestibility was depressed linearly as the amount of crushed wheat in the diet increased from below 10% to 36% of dietary DM. The lowest pH for any individual cow during a 24 h period was 5.4, and the amount of time that rumen fluid pH was <6.0 ranged from 0 to 14 h depending on the amount of wheat consumed (Leddin *et al*., 2009). Figure 2.12 shows these results.

With depressed ruminal pH, NDF digestibility reduced due to a shift in RMO composition (see Figure 2.12). This would result in a shift in VFA production from acetate to propionate (Van der Merwe & Smith, 1991; Firkins *et al*., 2001). At a low ruminal pH (<6), ruminal function is considered to be sub optimal (Dehghan-banadaky *et al*., 2007). McAllister *et al*. (1991) and Beauchemin *et al*. (1994) relates this to rapidly fermentable carbohydrates resulting in a sub 6 ruminal pH. Excess fermentation of starch to VFA in the rumen may thus overwhelm the buffering and absorptive capacity of the cow, leading to the reductions in ruminal pH.

A decrease in ruminal pH can decrease appetite (Britton and Stock, 1987), fibre digestion (Mould *et al*., 1983; Leddin *et al*., 2009) and microbial yield (Strobel and Russell, 1986), leading to decreased energy intake and lower animal production. Several studies have further shown that dry matter intake (DMI) decreased significantly when more rapidly available starch sources were fed (McCarthy *et al*., 1989; Moore *et al*., 1992; Aldrich *et al*., 1993).

With the use of highly fermentable carbohydrates such as wheat (Dunshea *et al.,* 2012ab) or large amounts of maize (especially low vitreous), the requirement to decrease the extent and rate of ruminal starch fermentation is therefore apparent. Under these conditions ruminal pH could drop below an optimal 6 and would impair animal performance due to either SARA or acute acidosis (Nocek, 1997).

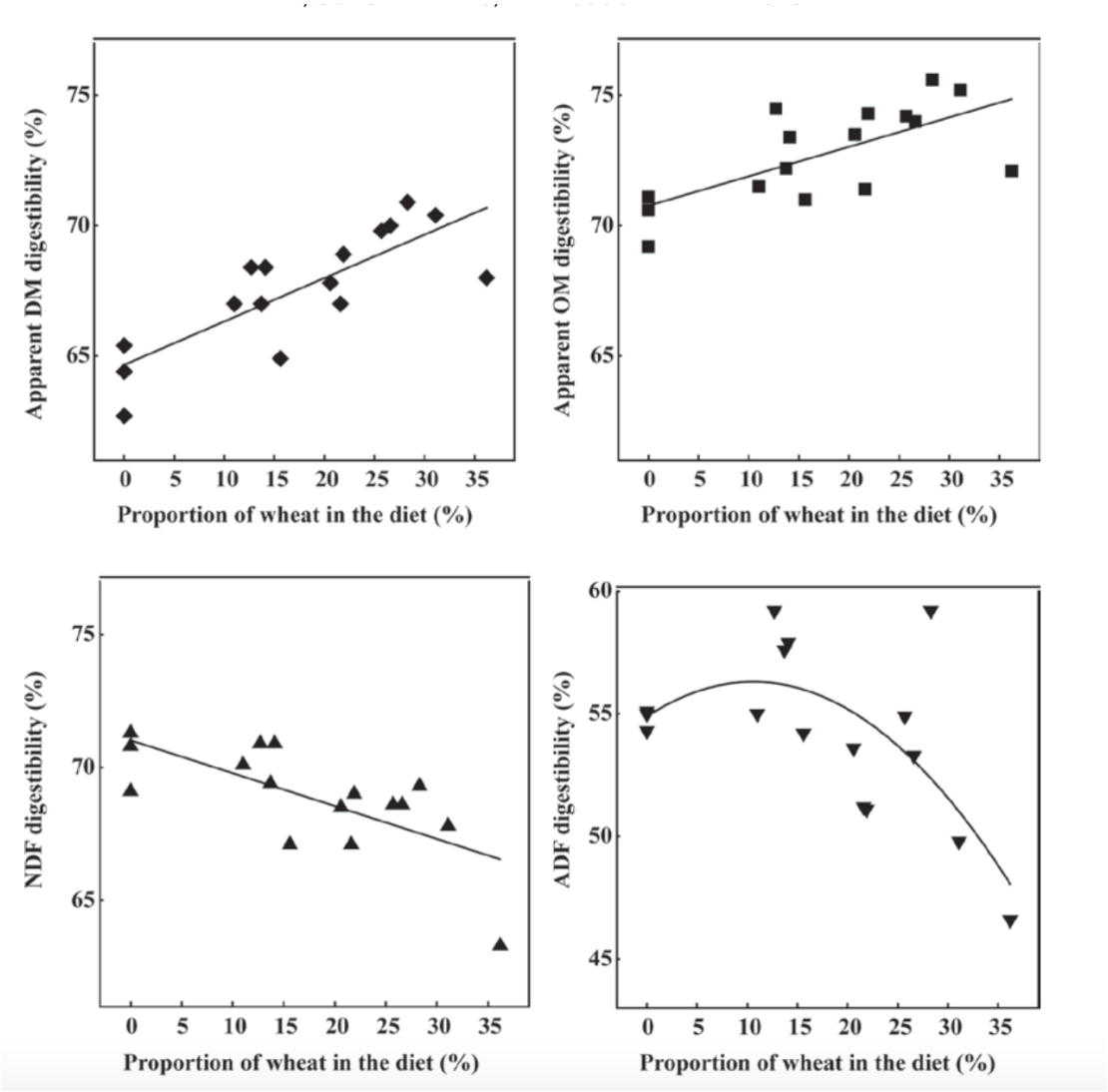


Figure 2.12. Effect of increased diet wheat on fibre digestion in dairy cows (Leddin *et al*., 2009).

2.9 Maize hardness tests

Various methods have been devised in an attempt to determine maize hardness. As ruminal and total tract starch digestibility is directly related to maize hardness (Firkins *et al*., 2001; Ngonyamo-Majee *et al*., 2008ab), it is generally accepted that animal performance changes according to hardness, thus the incentive to determine maize hardness accurately. These techniques range from chemical and physical, to more modern equipment. In this section a brief discussion of these techniques is conducted.

2.9.1 Direct methods

2.9.1.1 Chemical analysis

According to Mestres *et al.* (1991) a chemical analysis of maize to determine hardness involves the determination of:

- Dry matter (DM)
- Ash content
- Nitrogen (N)
- Ether extract

Due to the variation in zein content, the protein content (derived from N) might be used as an indicator for maize hardness (Robutti *et al.*, 1997). Blandino *et al*. (2010) further proposes that starch content, moisture and fibre are also related to maize hardness. Both Blandino *et al*. (2010) and Mestres *et al.* (1991) agree that correlations between ash content and density exist between maize of various vitreousness. Protein content proved to be an inconsistent indicator of maize hardness. While Mestres *et al.* (1991) found a good correlation between protein and maize hardness; in contrast, various other authors reported that hardness and protein content did not correlate (Paulsen and Hill, 1985; Robutti *et al*., 2000; Delcour and Hoseney, 2010). It can be seen from Table 2.2 that crude protein (CP) is essentially unrelated to genotype, stage of maturity starch content and vitreousness (McAllister *et al*., 1990). The inconsistent relationship between maize hardness and CP, suggests that methods to determine protein differ greatly and that in animal feed application zein should be tested, individually (Larson and Hoffman, 2008).

2.9.1.2 Physical analysis

This extremely laborious method involves the hand dissection of kernels into the vitreous and floury components followed by the weighing and the calculation of V:F ratios (Paulsen *et al.,* 1985; Dombrink-Kurtzman and Knutson, 1997; Gaytán-Martínez *et al.,* 2006). The method requires the visual examination of a selected kernel with the amount of vitreousness being proportional to the amount of vitreous endosperm. The reliability of this method is, however, dependent on observer experience and

competence (Paulsen *et al.*, 1985). Furthermore, Louis-Alexandre *et al.* (1991) showed repeatable results with the calculation of a vitreousness index of maize kernels based on the measurement of the vitreous and the total endosperm areas through a view of sectioned kernels. Due to variability of results (Blandino *et al.,* 2010), time constraints and the laborious nature of analysis, this method was not further considered.

2.9.1.3 Particle size index

The particle size index method is the most common method used to determine hardness (Fox and Manley, 2009) and involves the milling of a sample and then fractionating the ground material through a series of sieves. This test is known as the particle size index (PSI) test (Abdelrahman and Hoseney, 1984; Wu, 1992; Haddad *et al*., 1998) and has the benefit (if multiple sieves are used) that some information could be gained on the variation of hardness within a sample. In addition, the ratio between larger particle sizes and smaller ones can be calculated, thereby giving a coarse/fine ratio (a higher number indicate harder samples). Harder maize genotypes with more vitreous endosperm will break less easily and will thus accumulate more on the top sieves. Softer genotypes, containing flourier endosperm, will break more easily and tend to pass the sieves more readily (Guelpa *et al*., 2015a). Earlier PSI test inconstancy has been improved by a clearer understanding of milling methods with combination to multiple sieves vs. a single sieve as well as sieve mesh sizes (Fox and Manley, 2009). With a simple procedure, Abdelrahman and Hoseney (1984) predicted maize hardness by using a single sieve of 150 µm. In contrast pre-grinding of a sample through a 1 mm screen whereafter sieving the milled sample through a single 106 µm sieve have been shown to be an accurate indication of maize hardness (Burden, 2010; Cruywagen, 2016). Although Blandino *et al*. (2010) also reported that the resultant coarse:fine ratio derived by the PSI test are the most accurate method to predict milling quality, Wu (1992) warns that agglomeration could conceal results due of a relatively high oil content of maize. Due to the simplicity, accuracy, relative low cost, and speed of this method, it was selected as a possible method to quantify maize hardness as a regular measurement within the animal feed industry.

2.9.1.4 Other direct methods

The Tangential Abrasion Dehulling Device (TADD) entails a process where kernels

are abraded for a defined period of time. The amount of material removed from the kernel is calculated, with higher values indicating softer kernels (Fox and Manley, 2009). While the Stenvert test is a variation of the PSI test to determine maize hardness (Pomeranz *et al*., 1985), resistance to breakage can also be determined by both the Stein breakage tester and the Wisconsin breakage tester. Both the latter tests determine resistance to breakage and are based on the principle that breakage of hard genotype kernels is lower than that of the softer genotypes (Paulsen and Hill, 1985). Due to specific equipment requirements, these tests were not further considered for application in the animal feed industry.

2.9.2 Indirect Methods

Indirect methods to determine maize hardness are normally easier, cheaper and less time consuming than direct methods (Hoffman *et al*., 2010). An infinite number of sample analysis are also possible and can be automated (Fox and Manley, 2009).

2.9.2.1 Near infrared spectroscopy

Near infrared spectroscopy (NIR) is an example of an indirect testing method and has been used for more than 20 years to predict maize hardness (Osborn, 2006; Fox and Manley, 2009). These spectroscopic methods are not necessary kernel destructive and can be calibrated against an unlimited choice of reference methods. Infinite sample analysis of materials is also possible, without time-consuming practices such as sample preparation or interpretation of results. Light in the NIR region of the electromagnetic spectrum (750−2500 nm) is absorbed by overtone and combination vibrations of X−H bonds such as C−H, O−H, S−H, and N−H, which are abundant in organic molecules (Gustin *et al*., 2013). Therefore, NIR transmittance and reflectance profiles of biological materials are used to derive information about the quality and quantity of organic material within the sample.

With early studies, NIR transmittance spectra were obtained from whole maize kernels at wavelengths between 850 and 1050 nm. As this wavelength represented particle size differences, only 860 nm absorbance values were used to measure kernel hardness, (Robutti *et al.*, 2000; Lee *et al.*, 2006). In later studies, a wider wavelength range of 1000 to 2500 nm was used to obtain reflectance spectra of whole kernels (Pomeranz *et al*., 1985; Hoffman *et al*., 2010). A number of studies with a single

wavelength of 1680 nm were used in earlier studies to determine hardness of milled maize (Pomeranz *et al*., 1985). According to Fox and Manley (2009) these were based on early instrumentation in which filters were used, as opposed to current monochromators or band splitting technology. Williams (2009) further reported that although a number of wavelengths (starch and protein) are associated with hard or soft endosperm, 1680 nm is not associated with maize hardness. A single wavelength of 2230 nm has recently been shown to be accurate to determine maize hardness of milled samples (Guelpa, 2015). At a 2230 nm wavelength, reflectance is effectively independent of chemical information and varies only with regards to particle size difference with respect to the milled sample (Downey *et al*., 1986; Hoffman *et al*., 2010; Gustin *et al*., 2013; Guelpa, 2015). Therefore, at 2230 nm wavelength, the absorbance essentially determines the same information as directly measured with the PSI.

The scanning of bulk milled maize samples are further less complicated and more practical compared to that of single maize kernels (Mestres *et al*., 1995), therefore single kernel applications are less common than bulk milled calibrations. This is associated with a relatively high amount of variation found within single maize kernels due to the relatively large portion of germ present (Spielbauer *et al.*, 2009).

A recent advancement in NIR technology is the development of portable devices that greatly improves the practicality and versatility of NIR usage (Camps *et al.,* 2014). These hand held devices are smaller and lighter (device weight is +/-60g) than conventional NIR's. This microNIR can be used in reflectance as well as transmission mode and can be applied for either single kernel as well as milled bulk applications. It has been shown by Guelpa (2015) that these handheld devices are equally accurate as conventional NIR's to determine maize hardness. Therefore, the only advantage of the microNIR compared to the traditional NIR is practicality and ease of use.

Near infrared (NIR) hyperspectral imaging has been successfully used as a non-destructive advanced hardness method of determining maize kernels by three-dimensional images of whole kernels (Fox and Manley, 2009; Williams, 2009; Guelpa, 2015). Also, NIR hyperspectral imaging is a powerful spectroscopic technique, which is capable of capturing images at many wavelengths in the NIR region in both transmission and reflective modes. As this method is time consuming and requires data to be processed by trained individuals, hyperspectral imaging was not further considered.

The global animal feed industry however already uses NIR technology extensively as

a qualitative and quantitative analytical tool. Almost all modern animal feed factories employ NIR technology not only to ensure raw material quality, but also to determine rapid, accurate roughage analysis. Large investments in accurate calibrations of nutrient components have been made to ensure accurate analysis of both raw materials and roughage (e.g. lucerne hay and silages). The mere fact that NIR technology is already available, combined with ease of use, speed, and low cost and infinite application makes this method a very attractive method to determine maize hardness for the animal feed industry. NIR analysis was therefore selected as a possible method of determining maize hardness on a regular basis within the animal feed industry. To date accurate maize hardness NIR calibrations (required as reference) are nevertheless lacking and will need to be built.

2.9.2.2 Rapid Visco Analyzer

Rapid visco analyzer (RVA) is a method that relates biochemical components of maize to hardness (Fox and Manley, 2009). In essence RVA measures the viscosity developed with hydration and subsequent gelatinization of starch granules during heating and stirring in excess water (Almeida-Dominguez *et al*., 1997). It has been shown by various authors that the RVA can be used to quantify maize hardness (Yamin *et al*., 1999; Seetharaman *et al.,* 2001; Ji *et al*., 2003; Sandhu and Singh, 2007). Maize vitreousness determination rationale with RVA is summarized by Guelpa (2015b):

- Milled high vitreous maize has more coarser and less fine particles, compared to low vitreous maize (Almeida-Dominguez *et al.*, 1997).
- Coarse particles diffuse slower in water, limited swelling of the starch granules and slower viscosity development (Sahai *et al.*, 2001; Narváez-González *et al.*, 2006).
- The hydration rate of smaller particles is more rapid due to bigger surface areas, resulting in better gelatinization and higher a viscosity (Almeida-Dominguez *et al.*, 1997).
- Low vitreous kernels show a less prominent protein-to-starch adhesion effect compared to high vitreous kernels and therefore require less time to gelatinize (Almeida-Dominguez *et al.*, 1997).
- The thicker protein matrix of vitreous endosperm forms a barrier that slows hydration (Wang and Eckhoff, 2000) and gelatinization (Narváez-González *et al.*, 2006).

The viscosity (cP), temperature (°C), speed (rpm) and the heat:cool ratio are recorded every four seconds for each RVA test. The resultant curve is known as a viscogram and is being generated as a function of viscosity, temperature and time. Figure 2.13 illustrates this. According to Almeida-Dominguez *et al.* (1997), Seetharaman *et al.* (2001) and Narváez-González *et al.* (2006) harder genotypes reach peak viscosities earlier than soft genotypes. Harder genotypes further require a shorter time to reach peak viscosity in relation to softer genotypes.

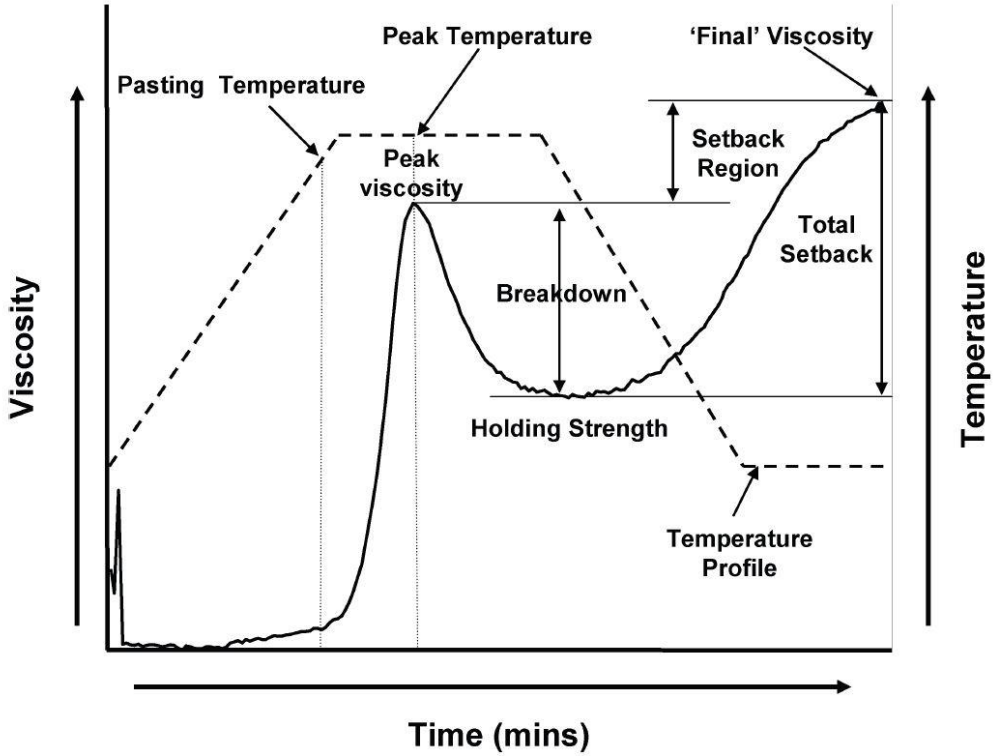


Figure 2.13. A Rapid Visco Analyser (RVA) viscogram (Agu *et al.*, 2006).

Despite requiring specific equipment, training and relative complex data processing, the RVA method was still selected due to accuracy, speed and relative low cost as a possibility for the determination of maize hardness within animal feed.

2.9.2.3 X-ray micro-computed tomography scanning (XCT)

Computed Tomography (CT) imaging also known as "CAT scanning" (Computed Axial Tomography) was invented in 1972 by a British engineer Godfrey Hounsfield of EMI Laboratories, England and by South African-born physicist Allan Cormack (Assmus, 1996).

X-ray micro-computed tomography (XCT) (high resolution CT) is a feasible approach to measure the density of various materials including individual maize kernels (Gustin *et al*., 2013; Singhal *et al*., 2013; Guelpa 2015; Guelpa *et al*., 2015b). X-rays traverse a cross section of the sample along straight lines; the intensity of the attenuated X-ray signal emerging is converted by a scintillator into light and recorded as a radiographic digital image (Gustin *et al*., 2013). Small angular steps rotate the object and the radiographic operation repeated each time. By scanning from various directions, projections of the object around 360° are collected. These X-ray shadows are processed to reconstruct a cross-section slice (Schena *et al.*, 2007). An X-ray radiograph image is essentially a chart of the linear attenuation coefficient of every point within the sample (Singhal *et al*., 2013). With 2-D visualization, X-ray densities are charted by black and white. An area of higher density appears white while lower density appears black (Singhal *et al.*, 2013).

X-ray micro-computed tomography utilizes X-ray attenuation from multiple radiograph “slices” of a sample to reconstruct a three-dimensional (3-D) representation of the structure (Gustin *et al.,* 2013). This 3-D image volume is reconstructed by using filtered back projection algorithms from the acquired 2-D data sets and thereby creating a 3-D digital virtual volume of the sample from the series of radiographs through tomographic reconstruction (Schena *et al.,* 2007). Figure 2.14 shows various digital images generated by XCT with two different maize genotypes (o2 = hard; N = normal). Specific, dedicated software packages such as VG Studio MAX software (Volume Graphics GmbH, Heidelberg, Germany) are available to analyze XCT data sets. The measurement of specific areas can be accomplished by using a region grower tool to identify selected grey value intervals.

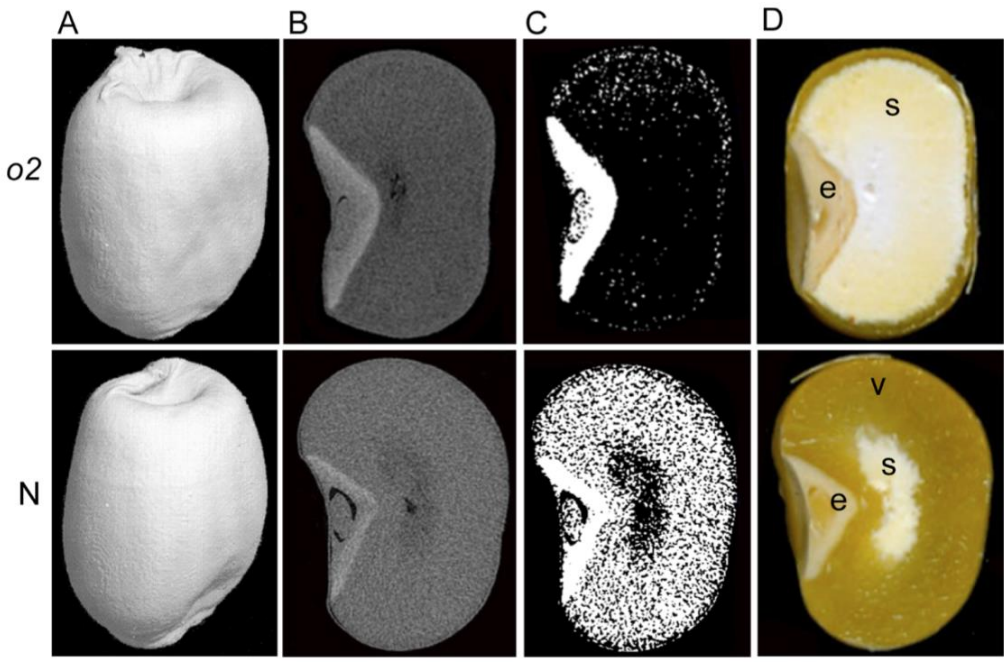


Figure 2.14. XCT analysis of a *o*2 mutant and normal (N) kernel from a segregating ear: (A) 3-D reconstruction; (B) single XCT section (gray scale shows relative density, with white indicating the highest density); (C) same section as in panel B with a lower threshold attenuation cutoff to remove signal from soft, starchy endosperm; (D) visible images of transverse hand sections through the same kernels showing embryo (e), vitreous (v), and starchy (s) endosperm at approximately the same location as the XCT section in panels B and C (Gustin *et al*., 2013).

As X-ray micro-computed tomography (XCT) is very accurate but expensive, time consuming and requires difficult analysis, it could be used as a reference method against other methods of interest.

2.10 Processing techniques

Maize starch is encapsulated in a hard pericarp, which is extremely resistant to microbial degradation in the rumen (Owens and Zinn, 2005). Most processing techniques in essence allows RMO easier access to the endosperm, thus increasing the rate of fermentation and VFA production (Theurer, 1986; McAllister *et al*., 1990). In contrast, where very high fermentable starch (Dunshea *et al.,* 2012ab) or very high

amounts (Leddin *et al*., 2009) of grain are fed, the objective of processing would be to decrease the rate and extent of ruminal fermentation in an effort to increase rumen resistant starch (RRS) and to decrease the risk of fermentative acidosis.

2.10.1 Background

Rumen resistant starch (RRS) is produced by rearrangements in the molecular structure of amylose that is generally less available than amylopectin (Fuller, 2003) and is the starch that escapes ruminal degradation while NRS is degraded in the rumen by the RMO to VFA's. Resistant starch is broken down to glucose in the small intestine by pancreatic enzymes. Therefore RRS could make a greater contribution to the glucose supply than NRS (Nocek and Tamminga, 1991; Rowe *et al.,* 1999; Fuller, 2003).

Various treatments developed to alter ruminal and total tract starch digestibility as well as site of digestion are thus investigated and includes cold and hot physical processing methods (Dehghan-banadaky *et al*., 2007), chemical treatment and more recently the use of enzymes (Gencoglu, *et al*., 2010; Weiss *et al*., 2011; Crosby *et al*., 2012; McCarthy, *et al*., 2013) and starch binders (Dunshea *et al*., 2012ab; Gonzalez *et al.,* 2014). The choice of processing technique will largely depend on storage, required speed of processing and cost (Dihman *et al*., 2002). Effectiveness of the processing technique will also determine the preferred processing technique. An objective to decrease (increase RRS) or increase (decrease RRS) ruminal starch fermentation will further determine the choice of processing method.

2.10.2 Physical Processing

Physical processing essentially breaks the physical barrier of the hull and pericarp, thereby allowing access of RMO's and digestive enzymes to the nutrient rich endosperm within grain. With *in situ* data, Lykos and Varga (1995) showed a linear inverse relationship between particle size obtained after processing and ruminal starch fermentation of maize. Various cold and hot physical techniques to achieve altered ruminal starch fermentation are subsequently discussed.

2.10.2.1 Cold processing

Grinding, rolling and cracking, while not identical, are all methods where the objective is to break the seed coat, reduce particle size, and so increase the surface area for digestion thereby increasing the rate and extent of VFA production (McAllister *et al*., 1990; Firkins *et al*., 2001).

2.10.2.1.1 Grinding

Grinding by a hammer mill will reduce the size of grains as well as fracturing the outer layers of the kernel exposing more of the endosperm to degradation (Dehghan-banadaky *et al.*, 2007). Early classic work by Moe and Tyrrell (1976) clearly indicates the importance and advantages of grinding grain to enhance ruminal starch degradability. According to Galyean *et al*. (1981) grinding greatly increases the surface area available for microbial attachment, while rate of starch degradation in the rumen varies inversely with particle size of the grain (Rowe *et al*., 1999; Dehghan-banadaky *et al.*, 2007). Maize hardness (vitreousness) affects the response to physical processing. The harder the grain is, the more damage occurs to the starch granules during processing and it also becomes more prone to shearing and shattering than is the case with softer grains where the starch granules tend to remain intact (Rowe *et al*., 1999). In a study evaluating particle size of dry milled maize (4.8, 2.6 and 1.2 mm), Callison *et al*. (2001) reported that fine grinding of maize greatly increased ruminal starch digestibility. Decreasing the particle size of maize affected true ruminal digestibility of NSC quadratically (49.8, 46.5, and 87.0%, respectively) (Callison *et al*., 2001). Total tract starch digestibility improved only marginally (91.3% vs. 98%) because of compensatory digestion post ruminally (Callison *et al*., 2001). In the same study, decreasing the particle size of the grain, increased the rate of NSC digestion in the rumen and the 1.2 mm particle size decreased ruminal pH compared to the 2.6 mm particle size, but milk fat percentage and yield were not affected. In a review of maize processing, Firkins *et al*. (2001) noted that increased NSC digestibility corresponded with decreased NDF digestibility in the rumen. Similarly, total tract digestibility of starch (Knowlton *et al*., 1996; Yu *et al*., 1998) or NFC (Wilkerson *et al*., 1997) was improved by fine grinding of dry maize (Eastridge, 2006; Eastridge *et al*., 2011). Thus, an optimal amount of rumen-degraded starch could maximize the total digestibility of carbohydrates (NSC plus NDF). The positive effect of mechanical processing will be greater with starch sources with lower ruminal degradability of starch

(e.g., sorghum > maize > barley > wheat) (Eastridge, 2006). A rapid decrease of ruminal pH with a subsequent lowering of NDF digestibility associated with acidosis is the main reason for this (Eastridge, 2006).

2.10.2.1.2 Dry rolling and cracking

Dry rolling is the process where grain kernels pass through rotating rollers thereby rupturing the pericarp and exposing the endosperm to microbial action (Dehghan-banadaky *et al.*, 2007). Dehghan-banadaky *et al.* (2007) further emphasizes that rolling produces more uniform particle sizes with less fine particles than grinding. Improved feed efficiency (Economides *et al.,* 1990) was reported with rolled vs. whole grain while acidosis risk will be lower than with grinding due to less fine particles and thus reducing the rate of fermentation (Rowe *et al*., 1999; Eastridge *et al*., 2011). In a study to determine the effect of particle size and site as well as the extent of starch digestibility, Rémond *et al*. (2004) in concurrence found that rumen starch digestibility changed from 70% with finely milled maize compared to 54% with coarsely rolled maize. Although maize particle size did not affect small intestinal starch digestion significantly, a tendency was found towards higher digestibility with larger particles (Callison *et al*., 2001; Rémond *et al.*, 2004). Irrespective of maize type, total tract starch digestibility significantly decreased with increased particle size (Rémond *et al.*, 2004).

2.10.2.2 Hot processing

Hot processing techniques either use heat, moisture and pressure or a combination thereof (Dehghan-banadaky *et al.*, 2007). During the early 1970's, Waldo (1973) reported that when heat and moisture are added in a combination to grain by means of steam rolling or flaking, starch gelatinizes and may increase degradation by RMO's (Van Soest, 1994). Subjecting grain to moisture, pressure and heat makes the starch granules more accessible for bacterial attachment (Huntington, 1997) and thus also for ruminal fermentation and enzymatic digestion in the intestine (Nocek and Tamminga, 1991). Van Soest (1994) warns that excessive hydrothermal treatment could result in lower starch digestibility due to denaturation of the protein and protein-carbohydrate condensations (Mailard reaction) and also possible caramelization of carbohydrates. He concludes that this might be one of the reasons for conflicting results in research involving hydrothermal treatment of maize. According to Van Soest

(1994), the best improvement is associated with the gelatinization and rupture of the starch granules (achieved by hydrothermal treatment), possibly due to limited re-association via retro gradation by the branched structure of the polysaccharide.

Despite processing risks and inconsistency, it is generally accepted that starch is more available in the rumen when heat processed.

2.10.2.2.1 Steam processing

Steam is the most common method of hot processing and involves the application of steam for 3 to 5 minutes in a space above the roller mill prior to rolling or flaking of the grain (Dehghan-banadaky *et al.*, 2007). Low pressure flaking is achieved by exposing maize to low pressure steam for 30 to 60 minutes, attaining temperatures of 95 to 99 $^{\circ}$C, with the moisture content increasing to 150 to 200 g/kg. High pressure, in contrast involves the use of a pressure cooker in which the grain is subjected to moist steam at a pressure of about 3.5 kg/cm^2 for approximately 3 minutes. The heated grain is then allowed to cool to 95 to 99 $^{\circ}$C before rolling (Dehghan-banadaky *et al.*, 2007).

Similar to particle size results, Callison *et al.* (2001) reported a 20% increase in true NSC digestibility in the rumen with steam rolled coarse ground maize compared to non treated coarse ground maize. Total tract starch digestibility in contrast was unaffected (Callison *et al*., 2001) between treatments. These results therefor indicate increased ruminal starch fermentation with heat treatment, and increased compensatory post ruminal starch digestibility of untreated maize, resulting in similar TTSD. In a study investigating steam flaking of sorghum Chen *et al*. (1995) also reported increased total tract DM, OM and starch digestibilities (63 vs. 57%; 66 vs. 59%; 98 vs. 83% respectively) with milled vs. steam flaked sorghum. Similarly, Dihman *et al*. (2002), in a study investigating maize processing, concluded that the digestibility of starch increased with 6 and 3 percentage units, respectively, by feeding steam flaked maize compared with coarse and finely ground maize. Cows fed steam flaked or fine ground maize produced 4% more milk with lower fat content compared with coarse ground maize (Dihman *et al*., 2002). Corona *et al*. (2006) further reported increased VFA concentrations in the rumen for steam flaked compared to dry rolled maize diets.

It is thus generally accepted that the addition of steam increases ruminal starch degradation and VFA production when compared to dry processed maize and the

effects have been well documented (Theurer, 1986; Fiems *et al*. 1990; Chen *et al*., 1994; Van Soest, 1994; Owens *et al*., 1997; Knowlton *et al*., 1998; Santos *et al*., 1998; Yu *et al*., 1998; Rowe *et al*., 1999; Callison *et al*., 2001; Dihman *et al*., 2002; Corona *et al*., 2006; Dehghan-banadaky *et al.*, 2007). Dehghan-banadaky *et al.* (2007) concludes that the improvements observed with steam flaked processed maize are due to the gelatinization of starch granules and the subsequent disruption of the protein matrices, thus making the starch more susceptible to amylolytic attack.

2.10.2.2.2 Pelleting

Pelletizing is achieved by forcing ground grain through a die using a roller with or without steam application (Dehghan-banadaky *et al.*, 2007). The starch is partially gelatinized by the heat and steam (increased moisture to 10-12%) used in the conditioning process (60-83°C) as well as by the friction generated as the feed passes through the die (Rowe *et al*., 1999).

Gardner *et al*. (1997) reported higher rates and extent of ruminal starch digestion for cows fed pelleted diets compared to dry ground diets. In contrast, Svihus *et al*. (2005) suggested that steam conditioning and pelleting gelatinize only 10–200 g/kg of starch and will not have a marked effect on either ruminal starch degradability or physical quality of the feeds. According to Dehghan-banadaky *et al.* (2007) this data suggest that the particle size reduction effect of pelletizing is responsible for the increased ruminal starch degradability as observed by some researchers. Table 2.5 shows the differences in ruminal pH, and VFA shift that occurs with pelleting vs. dry grounding of cereal grains.

From Table 2.5 it is clear that pelleting will increase ruminal starch degradability as can be seen from the lower ruminal pH values and the higher propionic to acetic acid ratio with pelletizing compared with dry grounding (Ørskov, 1986). The effect observed might thus be attributed to the particle size reduction effect of pelleting and not pelleting per se.

Table 2.5. The effect of processing of different cereals on rumen pH, proportion of acetic and propionic acid (Ørskov, 1986).

			Molar proportion of:	
Cereal	Process	Rumen pH	Acetic acid	Propionic acid
Barley	Whole	6.4	52.5	30.1
	Ground pelleted	5.4	45.0	45.3
Maize	Whole	6.1	47.2	38.7
	Ground pelleted	5.2	41.3	43.2
Oats	Whole	6.7	65.0	18.6
	Ground pelleted	6.1	53.2	37.5
Wheat	Whole	5.9	52.3	32.2
	Ground pelleted	5.0	34.2	42.6

One of the main reasons for pelleting is to ease feed handling. Pellets generally flow better than meal through bins and augers used on farms, feed factories and delivery vehicles. Another typical reason for pelleting feeds is to improve palatability by reducing fine material. This may be important when grains are not fed in a total mixed ration (TMR). Pellets further help to reduce ingredient separation in a complete feed, while increasing density.

Although pelletizing offers an easy, cost effective and practical mechanism for controlling rate and site of digestion (Rowe *et al*., 1999), results on the effects of pelleting on nutrient digestibility in the rumen or whole tract are not consistent in literature.

2.10.2.2.3 Other hot processing methods

Various other techniques are used to enhance starch utilization. Extrusion and expanding are widely used in monogastic application, but little work has been done relating to ruminant nutrition (Dehghan-banadaky *et al.*, 2007). Extrusion cooking is a process that was developed to gelatinize cereal starch, where the principle is to grind, add moisture, heat and pressure by forcing the grain through a die (Dehghan-banadaky *et al.*, 2007). The temperatures of extrusion are high (125-170°C); however, there is a relatively short time (15-30 seconds) at these high temperatures (Rowe *et al*., 1999). It has been shown by Shabi *et al*. (1999) that extrusion could increase post ruminal digestibility of NSC without any changes in ruminal VFA production. Expansion by popping in contrast, involves exposure of the kernels to 230-240°C for 30 seconds

(Van Soest, 1994). This causes the grains to expand approximately 1.5 to 2 times their original volume with rupturing of the pericarp (Van Soest, 1994). By using rumen evacuation techniques, Tothi *et al*. (2003) showed in a study investigating the use of expanding, that both ruminal (81 vs. 85%) and total tract (84 vs. 96%) digestibility of maize starch could be improved compared to ground maize.

2.10.3 Chemical processing

Chemical treatment involves direct application of a concentrated chemical solution to grain several hours or days prior to feeding (Dehghan-banadaky *et al.*, 2007). Most of the chemical processing techniques has a similar effect as grinding and rolling as they aspire to allow greater access of the RMO's and digestive enzymes to the grain endosperm (Rowe *et al*., 1999; Dehghan-banadaky *et al.*, 2007). In contrast, other techniques aim to decrease the rate and extent of ruminal starch fermentation in an effort to decrease the risk of ruminal acidosis associated with the feeding of high amounts of highly fermentable starch.

2.10.3.1 Sodium hydroxide (NaOH)

The beneficial effect on starch digestibility of ruminants with the NaOH treatment of cereal grain has been long recognized (Archibald, 1924). Application of NaOH to barley or wheat grain, usually at 30-40 g/kg of grain DM, increases whole tract digestibility of the grain by destroying the seed coat (Dehghan-banadaky *et al*., 2007). In a study by Sriskandarajah *et al*. (1980) where NaOH treated barley was fed to grazing lactating dairy cows, the risk of acidosis with NaOH treated barley was found to be less than with grinding. The authors concluded that this was due to slower ruminal starch fermentation rates (Sriskandarajah *et al.,* 1980). These results are supported by those of De Campeneere *et al.* (2006). The latter authors reported significantly reduced *in situ* ruminal starch fermentation with NaOH treatment of wheat compared to untreated wheat. However, they reported no *in vivo* differences in total tract starch digestibilities. NaOH treated wheat resulted in increased milk yield and yield of fat and protein corrected milk compared to untreated wheat and rolled wheat (De Campeneere *et al.,* 2006; Degirmencioglu and Karabulut, 2010). With *in vitro* gas production, Gonzalez-Rivas *et al.* (2016) further reported that NaOH treatment (30 g/kg) of wheat decreased the rate of ruminal fermentation without decreasing total volume of starch

fermented compared to untreated wheat. These results indicate that NaOH treatment of small grains could either play an important part in the sifting of starch digestion from the rumen to lower in the digestive tract (De Campeneere *et al.,* 2006) or the slowing the ruminal rate of starch degradation without decreasing the amount of ruminal starch fermented (Gonzalez-Rivas *et al.,* 2016). Other researchers, in contrast, found no significant benefits of NaOH treatment of barley compared to rolled (Mulligan *et al.,* 2004) or ground (Greenlagh *et al.,* 1980; Ørskov, 1981) barley. Despite reports of higher *in situ* dry matter degradability with the treatment of maize with various dosage rates (0, 2, 4 and 6%) and curing times (1, 12, 24 and 48 hours), NaOH treatment of maize is not as effective as with small grain varieties (Berger *et al.,* 1981). The latter authors hypothesized that the poorer effect of NaOH treatment of maize is related to the relative slower fermentability of maize compared to wheat and other small grains. This is due to unique characteristics and properties of maize starch. The strong resistant protein matrix and relative large amylose content in maize compared to small grain affects fermentability by limiting microbial access to starch granules (McAllister *et al.,* 1993; Huntington, 1997).

Despite the benefits of NaOH treatment of predominantly small grain varieties, the practical application may not be feasible because of human health concerns regarding the handling of this corrosive chemical, the possibility of long term incidence of kidney lesions (nephritis) in dairy cows, and issues related to soil salinification (Dehghan-banadaky *et al.*, 2007).

2.10.3.2 Ammonia/Urea

With this processing technique a solution containing urea or ammonia is sprayed onto grain (mainly barley), and it is allowed to soak for several weeks (Dehghan-banadaky *et al.*, 2007). Dosing concentrations vary between 0.5 and 1% (Yaremcio *et al.,* 1991). Although various researchers reported positive results, this process is not practical in commercial use; hence it is not further researched in this study.

2.10.3.3 Exogenous enzymes

Van Soest further (1994) empasizes that starch from cereal grains can only be degraded by amylolytic enzymes if the pericarp has been ruptured thus exposing the amylose and amylopectin for hydrolizing.

Although the concept of grain processing with the addition of exogenous enzymes is not new, the concept is predominantly used to enhance digestibility of starch in monogastic species (Beauchemin and Rode, 1996; Rowe *et al*., 1999; Dehghan-banadaky *et al.*, 2007). Almost 70 years ago, Hastings (1946) recognized the importance of the application of exogenous amylase in poultry diets.

However, recently much work on exogenous enzymes and combinations thereof with specific application to ruminants have been conducted. In a recent review Sujani and Seresinhe (2015) highlighted the potential benefit of the use of enzymes in ruminant nutrition. Most of the ruminant work cited has been done with the addition of fibrolytic enzymes and the subsequent positive effect on DM, NDF and CP digestibilities (Rode *et al*., 1999; Officer, 2000; Bowman *et al*., 2002; Van der Vyver and Useni, 2012). Amylolytic enzymes have received little attention as a grain treatment, even though ruminant animal performance can be improved with a mixture of external enzymes (Beauchemin *et al*., 1999; Mora *et al*., 2002; Rojo, *et al*., 2005). Some exogenous enzymes are resistant to degradation in the rumen and have the potential to increase the digestibility of feeds, and in turn improve animal performance (Hristov *et al*., 1998; Klingerman *et al*., 2009). Because of its hydrolytic action, supplemental α-amylase may increase the availability of starch hydrolysis products in both the rumen and the small intestine (Tricarico *et al*., 2008). Klingerman *et al*. (2009) reported that α-amylase enzyme formulations had a relatively stable α- amylase activity in a 24-h *in vitro* ruminal fermentation study. These results suggest that the enzymes were not subject to extensive degradation by rumen microbes (Klingerman *et al*., 2009). In another study, McCathy *et al*. (2013) evaluated the feasibility of pre incubation of maize with exogenous amylase before either feeding or incubating it. Both *in vivo* and *in vitro* starch disappearance increased with maize that was pre treated with exogenous amylase, but this did not translate into improved milk yield (McCathy, 2011; McCathy *et al*., 2013). The authors attributed this to the relatively high energy content of the experimental diets compared to the cow's requirements (McCathy *et al*., 2013). This suggests that starch levels need to be relative low compared to cow requirement for exogenous amylase to have a significant effect on starch digestibility. These findings

are in concurrence with similar results of Gencoglu *et al*. (2010) and Weis *et al*. (2011). In an *in vivo* study by Weis *et al*. (2011), NDF digestibility nevertheless increased by adding exogenous amylase. Table 2.6 shows the digestibility results.

In a study with lambs to determine the effect of starch digestibility of pre treated sorghum with α-amylase and glucoamylase, Rojo *et al.* (2005) reported linear reductions in DM, OM and starch intake ($P < 0.05$). Both ruminal starch degragation and total tract starch digestibility of DM, OM and starch increased quadratically ($P < 0.05$). Furthermore, total VFA and protozoa production further decreased linearly ($P < 0.01$), whereas lactate was increased quadatically with α-amylase treatment. According to Rojo *et al.* (2005) α-amylase treatment indicate increased ruminal starch degradation in ruminants when fed diets with high amounts of low digestible grains such as sorghum or high vitreous maize (Cerrilla and Martínez, 2003).

Table 2.6. Total tract nutrient digestibility by cows fed diets with different starch concentrations with and without added amylase (Weis *et al*., 2011).

	Low starch diet		High starch diet		
Item	- Amylase	+ Amylase	- Amylase	+ Amylase	SEM
DM (%)	62.5	63,1	64.2	64.3	0.57
OM (%)	63.2	63.8	64.9	64.9	0.58
Energy (%)	61.9	62.7	63.6	63.7	0.59
NDF (%)	49.2	51.2	50.1	50.7	1.33
Starch (%)	88.4	88.1	86.9	87.8	0.77
CP (%)	59.4	59.3	59.7	58.8	1.18
DE (Mcal/kg)	2.69	2.71	2.77	2.78	0.03

Starch main effect (P<0.05)
Emzyme main effect (P<0.07)
n = 24 observations (6 cows x 4 periods)

El-Kady *et al*. (2006) conducted an experiment with the use of cellulase, xylanase, α-amylase and polygalacturonase enzymes in diets for buffalo calves. Feed intake was unaffected by enzyme supplementation but significant increases ($P < 0.05$) in average daily gain, total body weight gain, feed conversion (kg DM/kg gain) and (kg TDN/kg gain) and TDN were reported.

Beauchemin *et al*. (1999) summarized contributing factors for early inconsistency with the use of exogenous enzymes in ruminants as follows:

- Diet composition
- Type of enzyme preparation used
- Amount of enzyme provided
- Enzyme stability
- Method of enzyme application

Despite inconsistency of results, and still not fully understood, supplementation of exogenous amylolytic enzymes to ruminant diets shows some feed efficiency, growth performance and production performance benefits (Officer, 2009; Sujani and Seresinhe, 2015).

As can be seen in Table 2.7, it is generally accepted that the benefits of amylolytic enzymes in ruminant nutrition are still largely unexplored and need much more research. The addition of amylase is known to increase intestinal digestion, but the effect on both ruminal and large intestinal (hindgut) fermentation is still largely unknown.

Table 2.7. Summary of the effect of various processing techniques on grain and digestive function (adapted from Rowe *et al*., 1999) Key: ? indicates that the effect of the treatment processes is currently unknown; + indicates a minor effect, ++ a moderate, and +++ indicates major effects on grain and structure or digestion).

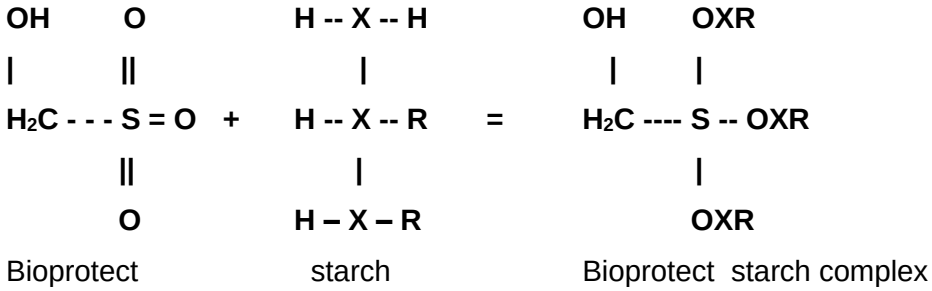
Process	Disrupts seed layer and/or exposes endosperm	Reduces particle size	Seperates starch granules and/or disrupts endosperm matrix	Disrupts starch granules and/or causes hydration and gelatanization	Processing increases		Improves overall		
					Fermentation rate	Intestinal digestion	Cattle	Swine	Poultry
Dry rolling	+++	+			++	+	++	+	
Grinding/milling	+++	+++			++	+		++	++
Steam flaking	+++	++	+	+	+++	++	+++	+++	++
Extrution	+++	-	++	+	++	++	+++	+++	++
Pelleting	+++	-	+	+	+	++	+++		++
Micronisation	+	+	?	?	?	++	++	?	?
Popping	++	-	?	+++	?	+++	?	?	?
NaOH whole grain	+		?	?	+	++	+	?	?
NaOH ground grain			?	?	?	?		?	?
Enzymes									
Amylase					?	++	+	+	+
Glucanase			?	?	?	++	?	++	++
Arabinoxylanase			?	?	?	++	?	++	++
Protease			?	?	++	?	?		

2.10.3.4 Starch binders

A commercial product (Bioprotect, RealisticAgri, Ironbridge, UK) has been shown (with in vitro gas production) to protect highly digestible starch, such as wheat, against ruminal degradation (Dunshea *et al.,* 2012ab). The active ingredient in these products is a stable non-volatile organic salt that forms complexes with the hydroxyl groups of starch at neutral or slightly acidic conditions (pH 6 to 7), as observed in the rumen (Nocek, 1997; Van Winden *et al.,* 2002). These complexes decompose under more acidic (pH 2 to 3) conditions such as in the abomasum and duodenum (Constable *et al.,* 2006), thus exposing the starch to be available for enzymatic digestion. Degradation in the lower intestine will be mainly driven by pancreatic α-amylase (Cerrilla and Martínez, 2003).

A typical starch can be represented as:

According to the suppliers, Bioprotect is in the form H_2CO-S (ONa)(OH) and has great affinity with the hydrogen bonds of starches. In mildly acidic conditions of the rumen (when highly fermentable carbohydrates are fed), complex Bioprotect starch structures are formed containing R-X-O-CH_2-SO_3 Na linkages. Alternatively, Bioprotect, containing three double bonded oxygen atoms, can form multi-links with a starch chain:



Dunshea *et al*. (2012a) conducted a study, using Bioprotect with *in vitro* gas production techniques, to determine if the amount of starch hydrolyzed in the rumen from wheat could be altered. The rate and extent of ruminal fermentation of untreated maize, untreated wheat (hard and soft) and starch binder treated wheat (hard and soft) were determined. Both hard and soft wheat were treated with 0, 4, 8 and 16 mL/kg of Bioprotect. The *in vitro* gas production parameters of both hard and soft untreated wheat were compared to that of untreated maize. Maximum amount of gas produced (Rmax) as well as rate constant (β) were recorded. Figure 2.15 indicates the rate of gas production (β) following the addition of Bioprotect to various wheat grains. A decreased rate of starch degradation (as indicated by the rate of gas production) was observed after treating wheat with the starch binder. Response maximized at 8 mL/kg (Dunshea, *et al*., 2012ab). In this study maize was used as a control.

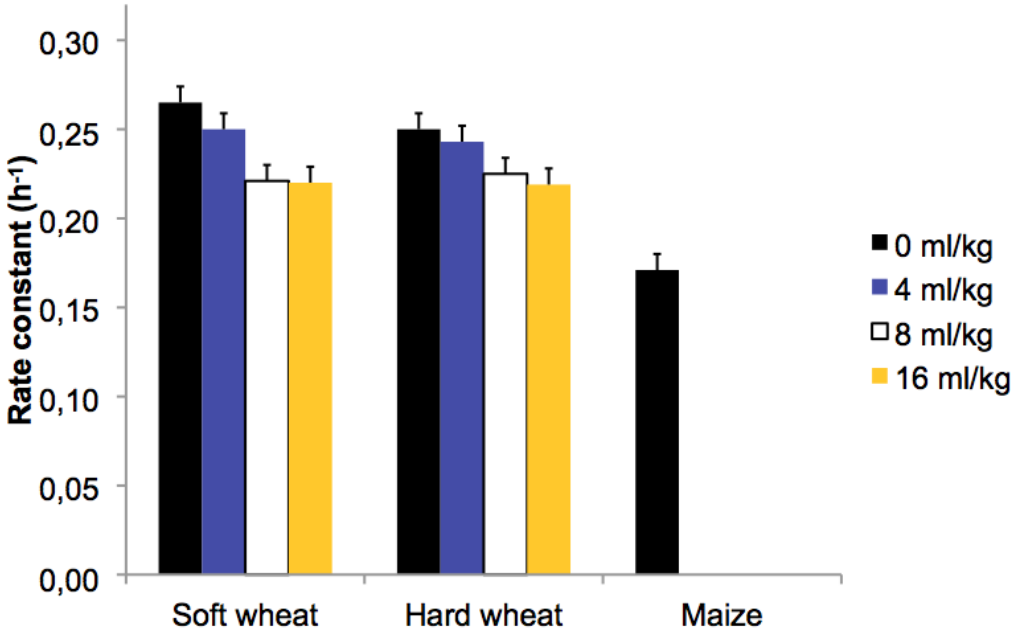


Figure 2.15. The effect of Bioprotect treatment level of various grains on the rate of gas production (β) (Dunshea, *et al*., 2012b).

Results of Dunshea *et al*. (2009b) indicate that wheat, irrespective of hard or soft genotypes, ferments faster than maize (Figure 2.15). The study further reported that hard wheat tends to have a lower β than soft wheat, while maize has a much lower β

than wheat (Figures 2.16 and 2.17). This data indicates a slower rate of gas production (and fermentation rate) for hard wheat (Dunshea *et al.,* 2012a). The rate of fermentation (as measured by β) of maize was significantly ($P < 0.001$) slower than for both hard and soft wheat (Dunshea *et al.,* 2012ab). Herrera-Saldana *et al.* (1990) and Huntington (1997) have also shown with in vitro degradability work that despite the higher starch content of maize compared to wheat, the rate of degradation is significantly slower (6.4 %/h vs. 23.5 %/h from 0-60 min incubation time). Although the impact of the starch binding agent on hard vs. soft wheat was reported, the impact on different genotypes of maize was not evaluated.

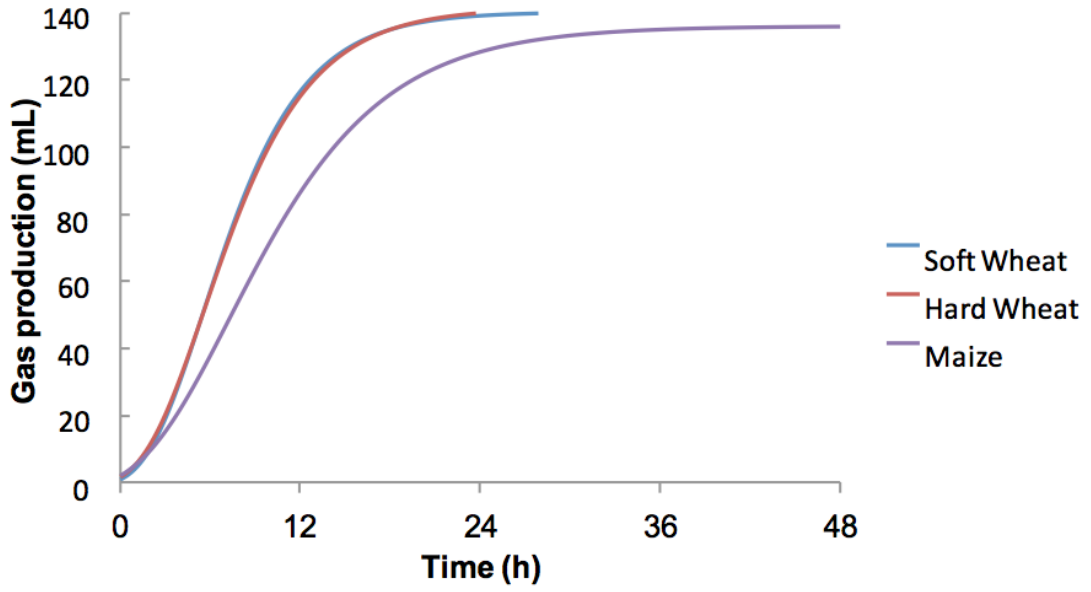


Figure 2.16. Differences in gas production between wheat and maize (Dunshea *et al.*, 2012b).

A study by Gonzalez *et al*. (2014) further evaluated the impact of Bioprotect on ruminal and total tract starch digestibility of lambs fed various cereal grains. No difference in total tract starch digestibility between treated and untreated wheat was reported (Gonzalez *et al*., 2014). The authors attribute this to sufficient enzymatic starch digestion in the small intestine (Gonzalez *et al*., 2014). Gonzalez *et al*. (2014) concluded that the use of a starch binding agent provides confidence that rumen protection of wheat starch still allows for enzymatic starch digestion in the small intestine. The authors nevertheless recommend further *in vitro* and *in vivo* research to confirm their hypothesis (Gonzalez *et al*., 2014).

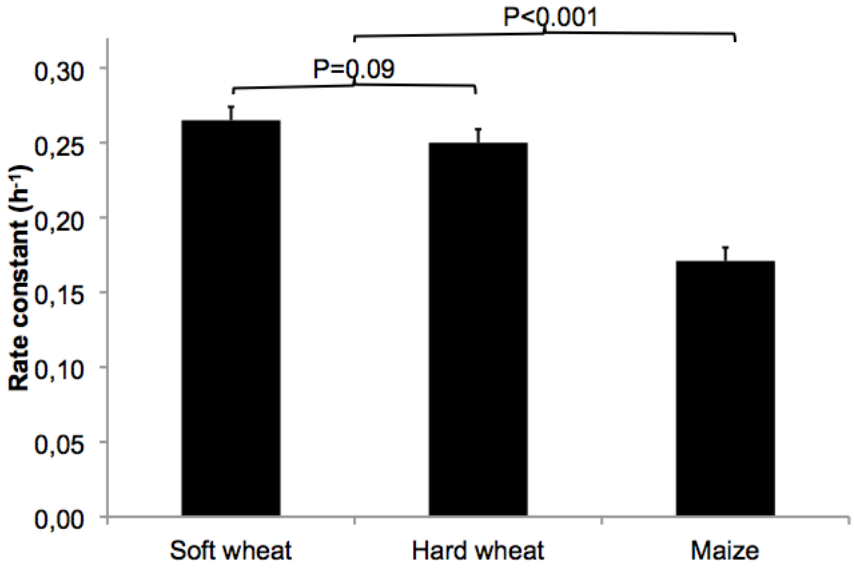


Figure 2.17. The rate of gas production for hard wheat tends to be lower than for soft wheat while maize has much lower β than wheat (Dunshea *et al*., 2012b).

Dunshea *et al*. (2012b) summarizes the effect of the use of starch binding agents in ruminant diets as follows:

- That the rate of fermentation of soft wheat is more rapid than that of hard wheat.
- That treatment of wheat with Bioprotect will decrease the rate of *in vitro* fermentation in a dose dependent manner with response maximised at 8 mL/kg.
- That the effect of a starch binding agent will be greater for soft wheat than for hard wheat.
- That the use of a starch binding agent can improve utilization of wheat by shifting the site of starch digestion from the rumen to the lower intestine.
- The use of a starch binding agent can reduce the risk of SARA when highly fermentable starches are fed to ruminants.

2.11 Summary

Maize is a major glucogenic supplier to high producing ruminants, but differs significantly according to vitreousness. High vitreousness of maize can lead to a negative effect on total tract digestibility and especially ruminal starch degradability and production of high producing lactating dairy cows. Vitreousness of maize is determined by the ratio of hard endosperm to soft endosperm in a kernel. Existing maize hardness determining methodologies are mainly used in food science to determine meal quality and grits production where high vitreous maize is preferred. In animal science either high or low vitreous maize, depending on requirement, is needed. A rapid, accurate, inexpensive and easy method to determine maize vitreousness within the animal feed industry to be applied to ruminant animals to date, however, is still lacking. Vitreousness of maize is determined by genotype, stage of maturity at harvest and environmental factors. Besides vitreousness, various processing methods of maize furthermore can change total tract and site of digestion. High producing animals require high amounts of starch to optimize production efficiency, but can often lead to metabolic problems, such as acidosis. Under such conditions it would be beneficial to decrease the rate and extent of ruminal starch fermentation. In order to utilize the high dietary amounts of highly fermentable starch efficiently without metabolic risk it would be beneficial to shift some of the digestion from the rumen to the small or large intestine. This would be needed without the loss of starch digested through the whole digestive total tract.

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CHAPTER 3

Evaluation of methods to determine maize (*Zea mays L*) quality and hardness (vitreousness)

3.1 Abstract

Maize (Zea mays L) forms a vital component of supplying carbohydrates to high producing dairy cows. Both ruminal and total tract starch digestibility of ruminant animals are significantly impaired by high vitreous maize compared to moderate floury or dent maize. Underlying genetic code, environmental conditions and stage of maturity collectively influences vitreousness of maize. Various methods including particle sieve index (PSI), near-infrared spectroscopy (NIR), rapid visco analyser (RVA) and x-ray micro-computed tomography (XCT) are currently exploited to determine maize hardness. Ninety maize samples mainly from South Africa, but also a few from Argentina and Ukraine were selected to be as diverse as possible according to vitreousness. Maize colour and cultivation method showed no influence with regard to hardness. Climatic conditions of origin showed significant differences between humid subtropical and cold semi arid production areas. Vitreousness of all 90 samples was first determined in Trial 1 by PSI, using a single 106 µm screen and NIR at a single absorbance of 2230 nm. Results indicated a significant relationship between the methods (r^2 = 0.7437 at $P \leq 0.01$). *Accurate vitreousness determination by both methods was established using specific intra-lab analysis. Based on the results of Trial 1, 10 hard samples and 10 soft samples were selected for Trial 2. These samples were used to evaluate the accurateness of maize hardness determination by means of three techniques, namely PSI, NIR and RVA. XCT methodology was used as a reference. PSI, NIR, RVA peak time and RVA peak viscosity all showed significant relationships to XCT. It was concluded that all the methods could be equally effective to determine maize vitreousness. While PSI and NIR are both practical, accurate, rapid and cost effective methods to determine maize vitreousness in the animal feed industry, neither XCT, RVA peak time nor RVA peak viscosity satisfied all requirements. As NIR technology is already available and exploited within the animal feed industry, it was concluded that NIR at a single absorbance of 2230 nm meets all the requirements of the animal feed industry to determine maize vitreousness.*

3.2 Introduction

Maize (*Zea mays L.*) is, at a global production of almost 1,1 billion tonnes per annum during the year of 2014 (FOA, 2016), the largest cash crop produced internationally and by far the most widely used energy source in ruminant feed (Dihman *et al*., 2002; Lopes *et al*., 2009). Maize is grown in most countries and utilized as human food, animal feed and in ethanol production (Ranum *et al*., 2014).

It was shown by various authors (Wolf *et al*., 1952; Robutti, 1995; Corona *et al*., 2006; Fox and Manley, 2009; Gustin *et al*., 2013; Guelpa, 2015) that maize hardness could be described by the ratio between the vitreous and floury endosperm. The higher the vitreous to floury endosperm ratio, the harder the kernel (Ngonyamo-Majee *et al*., 2008ab). Harder, vitreous endosperm is composed of densely packed starch granules embedded within a complex protein matrix, whereas the softer, floury endosperm contains larger, loosely packed starch granules (Gibbon *et al.,* 2003).

Unlike maize for the meal industry (human food), where hard maize is preferred due to higher yield and higher quality meals and grits production (Lee *et al*., 2007; Gustin *et al.*, 2013; Guelpa *et al*., 2015a), ruminant animals normally require softer maize. The negative effect on ruminant animal performance of high vs. low vitreous maize has been well documented (Firkins *et al*., 2001; Ngonyamo-Majee *et al*., 2008a; Allen *et al*., 2008; Hoffman and Shaver, 2009). Increased kernel vitreousness reduced ruminal *in situ* maize starch degradation (Philippeau and Michalet-Doreau, 1997; Correa *et al*., 2002; Ngonyamo-Majee *et al*., 2008ab). With ruminally and duodenally cannulated lactating dairy cows fed floury or normal dent endosperm dry maize, higher ruminal and total tract starch digestibilities were reported for the floury type of maize. (Taylor and Allen, 2005). Linear milk production increases were reported by Firkins *et al*. (2001) as total tract starch digestibility increased. When high amounts of highly fermentable starches are used it could be advantageous the decrease the rate of ruminal fermentation in order to limit metabolic disorders (Leddin *et al*., 2009).

Fox and Manley (2009) reviewed various methods to determine maize hardness. A very simple method is to fractionate a milled sample through a set of sieves; this is referred to as particle size index (PSI; Abdelrahman and Hoseney, 1984; Pomeranz *et al*., 1984; Pomeranz *et al*., 1986; Haddad *et al*., 1998). Milled maize genotypes, with more floury endosperm, tend to break easier and also pass a sieve more readily than milled harder genotypes (Abdelrahman and Hoseney, 1984; Guelpa *et al*., 2015). Generally, softer endosperm has smaller starch granules with higher amylopectin

content, thus a higher percentage of fine particles (Fox and Manley, 2009). While it is possible to determine a range of endosperm fractions by using a number of sieves, the relative size of the mill screen and the sieve used to sieve the milled sample is important (Fox and Manley, 2009). The milling of a sample through a 1 mm screen whereafter sieving the milled sample through a single 106 µm sieve has been shown to be an accurate indication of maize hardness (Burden, 2010; Cruywagen, 2016).

Near-infrared spectroscopy (NIR) is an example of an indirect testing method and has been used for more than 20 years to predict maize hardness, albeit more in the field of human food industry (Osborn, 2006; Fox and Manley, 2009). It has been further shown that NIR measurements of particle size in ground maize can be used as a hardness indicator (Pomeranz *et al*., 1986; Downey *et al*., 1986; Almeida-Dominguez *et al*., 1997, Guelpa, 2015). In early work with wheat a single wavelength of 1680 nm was used based on the correlation to PSI (Robutti, 1995). Williams (2009), in contrast, showed that a single wavelength of 1680 nm is not accurate to determine maize hardness. A wavelength of 2230 nm is of more interest in respect to milled samples where reflectance is effectively independent of chemical information and varies only with regards to particle size difference (Downey *et al*., 1986; Hoffman *et al*., 2010; Gustin *et al*., 2013; Guelpa, 2015).

When using rheological Rapid visco analyzer (RVA) curves, all the profile parameters (length, heating and cooling rates, holding time) would influence the results. According to Almeida-Dominguez *et al.* (1997), hard maize, compared to soft maize, contains predominantly coarser particles when milled. Coarse particles have slower water diffusion, limited swelling of the starch granules and slower viscosity development (Sahai *et al.*, 2001; Narváez-González *et al.*, 2006) while the smaller particles of softer maize have bigger surface areas that result in better and more rapid hydration, thus better gelatinization and higher viscosity (Almeida-Dominguez *et al.*, 1997). Soft kernels furthermore show a less prominent protein-to-starch matrix compared to hard kernels and require less time to gelatinize (Almeida-Dominguez *et al.*, 1997). The thicker protein matrix of vitreous endosperm thus forms a barrier that slows hydration (Wang and Eckhoff, 2000) and gelatinization (Narváez-González *et al.*, 2006) than that of floury endosperm.

Due to the differences in starch packing between vitreous and floury endosperm, kernel density is also a proximate measure of vitreousness (Gustin *et al.* 2013). Recently, X-ray micro-computed tomography (XCT or high resolution CT) was shown as a feasible non-destructive approach to measure the density of various materials

including individual maize kernels (Gustin *et al*., 2013; Singhal *et al*., 2013; Guelpa 2015; Guelpa *et al*., 2015b).

A global increased demand for cereal grains (FAO, 2016) necessitates increased dependency on higher grain production as achieved by modern GMO maize hybrids (Borlaug and Dowswell, 2003). While significant grain production increases were achieved (Borlaug and Dowswell, 2003), often the impact of changed kernel morphology on ruminant digestibility was overlooked (Owens, 2005).

As the global animal feed industry currently utilizes NIR technology to ensure raw material and product quality (Wrigley, 1999), it would be advantageous to employ this technology to predict maize vitreousness. Despite various methodologies shown to determine maize hardness, an easy, practical, accurate, rapid and cost effective method is required by the animal feed industry to quantify maize vitreousness. According to Manley (2014), many industries today approach NIR spectroscopy as the only viable alternative for quality control. The aim of this study was thus to evaluate NIR technology and compare it with other methods of maize hardness determination in order to establish NIR as a useful and rapid method to determine maize hardness in the ruminant feed industry.

3.3 Material and methods

3.3.1 General

Ninety maize samples of 1 kg each were collected throughout Southern Africa, Argentina and Ukraine. These samples originated from larger samples of the 2013 South African harvesting season that had been submitted to SAGL (Southern African Grain Laboratory) for regulation analysis, as well as from Ukrainian and Argentinian samples imported to South Africa during 2015.

The samples were selected to be as diverse as possible where considerations were:

- Method of production (irrigated vs. dry land)
- Genotype
- Climatic conditions during production
    - Annual rainfall
    - Ambient temperature

- Relative humidity

3.3.2 Sample preparation

Samples were milled through a standard laboratory mill (Scientec RSA Hammer mill Ser. Nr 372; Centrotec) to pass through a 1 mm screen. All milled samples were subsequently stored in air tight honey jars while intact kernel samples were stored in vacuum-sealed plastic bags. After preparation, all samples were stored at -18°C to prevent infestation. Samples were subsequently analyzed in two separate trials.

3.3.3 Starch analysis

All collected samples were analysed for starch content according to the method described by Hall (2009). These authors evaluated methods for starch analyses in animal feeds and proposed a method that ensures repeatable results (SD = 1.6 - 2.2) (McCleary *et al.,* 1997).

3.3.3.1 Various reagents and solutions are described by Hall (2009):

Preparation of acetate buffer (0.1M)

Sodium acetate buffer was prepared according to Hall (2009). To prepare 1 L of buffer solution, 6 g of glacial acetic acid (CH_3CO_2H) was dissolved in 850 mL distilled water. Solution pH was measured and adjusted with 1M NaOH to pH = 5, while continuously stirring on a stirrer plate. Upon reaching the desired pH of 5, distilled water was added to bring the solution to 1 L. Solution pH was always checked prior to use to assure a value of 5.

All other solutions required for starch analysis were commercially (MEGAZYME, Wicklow, Ireland) sourced and included:

- *Heat stable α-amylase*
- *Amyloglucosidase*
- *Glucose oxidase-peroxidase (GOPOD) reagent*
- *Glucose standard solution*

3.3.3.2 Starch analysis procedure

For starch analysis of maize, amounts of 0.25 +/- 0.01 g samples were weighed in duplicate into 50 mL plastic falcon tubes. Duplicate falcon tubes containing no samples with only reagents were carried through the entire procedure and used to subtract the absorbance value from the absorbance values of the samples.

A dispenser was used to first add 30 mL acetate buffer solution and then 300 µL *α*-amylase solution to the tubes. To immerse solid all tubes were vortexed. Tubes were subsequently placed in a boiling water bath (100 ^{o}C) vortexing at 10, 30 and 50 minutes intervals. The tubes were then cooled for 5 minutes in cold water (15 ^{o}C) to 50 ^{o}C. After 300 µL amyloglucosidase solution was dispensed in the tubes they were placed in a 50 ^{o}C water bath for 2 hours vortexing every hour. After removal from the water bath, the feed residue was transferred with dH_2O to 250 mL volumetric flasks, filtering through glass wool in a funnel. The assay was then brought to volume with dH_2O. After the flasks were shaken at least 25 times, sub-samples of 1mL were extracted and transferred to Eppendorf tubes. The tubes were subsequently centrifuged at 3000 rpm for 10 minutes. The contents of the tubes were thereafter transferred to U-bottom Lasec micro plates which was used for aliquots of 10 µL of the centrifuged samples, standards and blanks + 300 µL GOPOD solution. Glucose standards was prepared in duplicate by transferring 0, 2, 4, 6, 8 and 10 µL glucose standard solution into the wells of the micro plate with the addition of distilled water (dH_2O) to add a constant of 10 µL glucose standard solution + dH_2O so as to create blanks and increasing strength glucose solutions.

The prepared micro plate was subsequently incubated at 50 ^{o}C for 20 min wherafter D-Glucose concentration (g/L) using a spectrophotometer (Cecil CE 2021 2000 Series Lasec SA (Pty) Ltd) at absorbance of λ = 505 nm was determined within 30 min. after incubation.

3.3.3.3 Standard curve and calculation

Absorbance values of the reaction solutions minus the absorbance values of the reagent blanks were used in all calculations. The slope and intercept of obtained from standard regression equations were calculated and used the determine starch with the following equations (Hall, 2009):

µg glucose = absorbance (λ = 505 nm) x slope + intercept

Total starch + malto-oligosaccharide (%) = µg glucose x V x (1/1000) x (100/W) x (162/180)

Where:	V	= volume correction (0.1 mL taken from 250 mL = 0.04)
	100/W	= conversion to express as % starch
	162/180	= factor to convert from free glucose, as determined, to anhydroglucose (present in starch).

3.3.4 Hardness determination methodologies

In Trial 1, two methods to determine and rank maize vitreousness were compared, namely the PSI sieve at 106 µm and NIR hardness index at 2230 nm absorbance and the study involved all 90 samples.

In Trial 2, 10 soft and 10 hard maize samples were ranked and selected on the basis of hardness from data analyzed in Trial 1. These samples were then subjected to various other hardness methodologies:

- PSI sieve at 106 µm
- NIR at 2230 nm absorbance
- XCT
- RVAPV (Rapid Visco Analyser - Peak viscosity)
- RVAHS (Rapid Visco Analyzer - Holding strength)
- RVAFV (Rapid Visco Analyzer - Final viscosity)
- RVASV (Rapid Visco Analyzer - Setback viscosity)
- RVAPT (Rapid Visco Analyzer - Peak time)

3.3.4.1 Particle size index (PSI)

According to Abdelrahman and Hoseney (1984) and Guelpa *et al*. (2015), softer genotypes, containing flourier endosperm, will break easier when milled and tend to pass a sieve more easily. Generally, softer endosperm will have smaller starch granules with less amylose and higher amylopectin content, thus a higher percentage of fine particles (Fox and Manley, 2009). Results of Burden (2010) have further shown a 106 µm mesh screen to be an effective screen size to determine maize hardness.

Aliquots of 10g ± 0.01 g of all 90 milled samples were weighed in triplicate and sieved through a single 106 µm mesh screen sieve (Kingtest laboratory test sieve, Retsch GmbH, Series AS 200 basic, Germany). The portion of the sieved samples on the bottom pan was weighed (± 0.01 g) and the weights recorded. It was assumed that most of the soft endosperm would pass through the screen, while most of the vitreous endosperm would be retained on the top of the screen. The ratios of hard and soft endosperm (V:F) in relation to the whole kernel were then calculated. The 10 samples that had the highest V:F ratio will be referred to as “hard” maize, while the 10 samples that had the lowest V:F ratio will be referred to as “soft” maize. Digital images of the assumed hard (a) and soft (b) endosperm after sieving through a single 106 µm mesh screen sieve are presented in Figure 3.1.

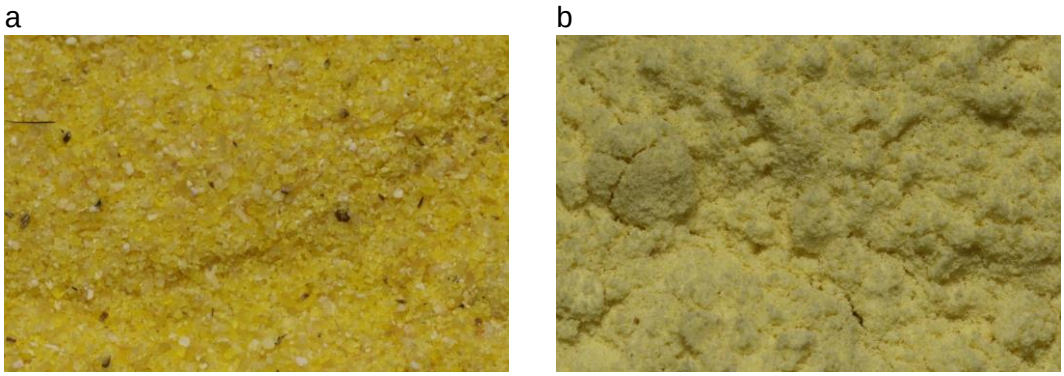


Figure 3.1. Digital images of hard (a) and soft (b) endosperm after sieving through single 106 µm mesh screen, taken with a Canon 7D Mk II SLR camera fitted with a Canon efs 60 mm macro lens.

3.3.4.2 Near-infrared spectroscopy (NIR)

When using NIR to determine maize vitreousness, Downey *et al*. (1986), proposed a method that utilizes a single wavelength (2230 nm), where reflectance is effectively independent of the composition of the samples, but only varies with regards to the milled particle size of the sample. In a study evaluating NIR, NIR hypespectral imaging, RVA and X-ray µCT techniques as a measure of maize hardness Guelpa (2015) reported similar positive results and concluded that maize hardness can indeed be effectively determined by NIR at a single wavelength of 2230 nm as proposed by Downey (1986). Guelpa (2015) concluded that the single absorbance of 2230 was as accurate as multiple wavelengths. Ninety maize samples were thus ground using a standard laboratory hammer mill (Scientec RSA Hammer mill Ser. Nr 372; Centrotec) fitted with a 1 mm mesh screen. A BÜCHI NIRFlex N-500 Fourier transform near-infrared spectrophotometer (BÜCHI Labortechnik GmbH, Flawil, Switzerland) with NIRLabWare (version 3.0) (BÜCHI Labortechnik GmbH, Flawil, Switzerland) near infrared (NIR) measurement software was used in diffuse reflectance mode to perform the measurements of the ground maize samples. The samples were presented in duplicate to the instrument in rotating glass Petri dishes. The NIR spectra were collected from 1000 to 2500 nm (9090-4000 cm^{-1}) at an optical resolution of 32 cm^{-1}, thus creating vectors of 2500 data points per scanned sample (done in duplicate). The raw spectra (no pre treatment) were used to measure the absorbance (log 1/R) at 2230 nm.

Hardness values were derived by the following log equation (Downey *et al*., 1986):

Hardness index = a + b(log 1/R)

Values for a = 40 and b = 100 were selected arbitrarily to produce a scale of hardness (Downey *et al*., 1986) from 0 to 26 and R = absorbance at 2230nm. Hardness index values for all samples were accordingly calculated.

3.3.4.3 Rapid Visco Analyzer (RVA)

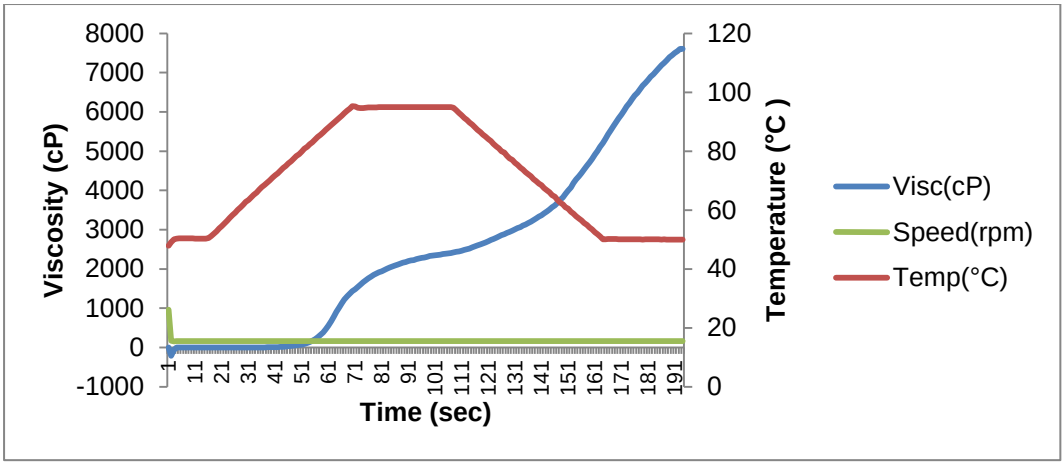
Twenty of the initial 90 maize samples were selected based on ranking data collected from both the PSI test and NIR analysis in Trial 1. Ten hard and 10 soft samples were selected and subjected to RVA analysis in duplicate. All samples were milled through a standard laboratory mill (Scientec RSA Hammer mill Ser. Nr 372; Centrotec) to pass

through a 1 mm screen. Accurate digital moisture determination was done in duplicate at 120°C by a Radwag moisture analyser (NDC Technologies, Irwindale, California, USA. Model Max50/NH). Pasting properties of maize were then determined using a Rapid Visco Analyzer (RVA) (Perten Instruments, Model 4500, Australia). Distilled water (25 ± 0.01 g) was added to the milled maize (5 ± 0.01 g) in an aluminum RVA canister to obtain a total constant sample weight of 30 ± 0.01 g. The masses of the dH_2O and maize were adjusted (± 0.01 g) to compensate for the differences in moisture content of each sample. In all the tests a moisture level of 15% was maintained, resulting in a relatively high solid percentage. Stirring with a plastic paddle prevented clumping after which a pre-programmed profile was initiated. Results by Guelpa (2015) showed that any RVA profile could be used to predict the hardness of maize samples, as the use of different RVA profiles did not influence results. Rheological information (RVA curves) was thus captured by means of a standard profile (AACC, 1999). The respective holding time, heating rate and final temperature used in this study are presented in Table 3.1.

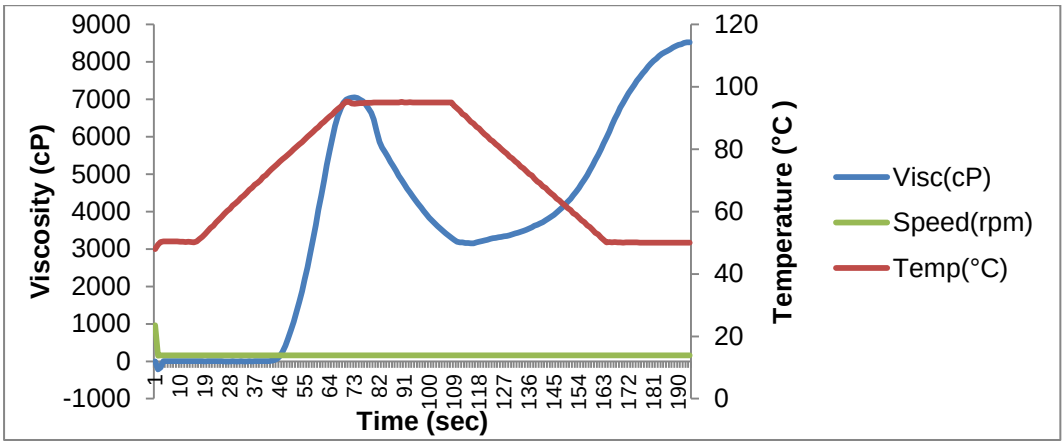
Table 3.1 Details of the RVA maize profiles (temperature and time).

Stage	Standard profile
Initial temperature (°C)	50.00
Initial holding time (min)	2.00
Heating time (min)	3.42
Max temperature (°C)	95.00
Hold at max temperature (min)	2.30
Cooling time (min)	3.48
Final temperature (°C)	50.00
Final holding time (min)	2.00
Total test time (min)	13.00

For each of the tests; viscosity (cP), temperature (°C), speed (rpm) and the heat-cool ratio were recorded every four seconds, therefore generating three measurement vectors of 193 data points per sample. The resulting curve, reporting the viscosity and temperature ramp as a function of time, is called a pasting curve or viscogram. Clear differences in viscograms of the selected hardest (a) vs. the softest (b) maize sample are apparent in Figure 3.2.



a



b

Figure 3.2. Recorded viscograms of the selected hardest (a) and the softest (b) selected maize samples.

3.3.4.4 X-ray micro-computed tomography (XCT)

The same 20 samples (10 hard and 10 soft) as selected for RVA analysis were used to perform XCT analysis. Consequently, 150 maize kernels were arbitrary selected from the 10 soft samples (15 kernels per sample), and 150 kernels from the 10 hard samples (15 kernels per sample). Florist oasis square discs (+/-10 cm x 12 cm x 2 cm) were prepared in order to facilitate simultaneous XCT scanning of multiple kernels. The low density of the florist oasis provided for clear distinction from the subjects of interest and was therefore a suitable medium for mounting purposes. From the 10 soft samples, 15 kernels were placed without touching each other in each of 10 florist oasis

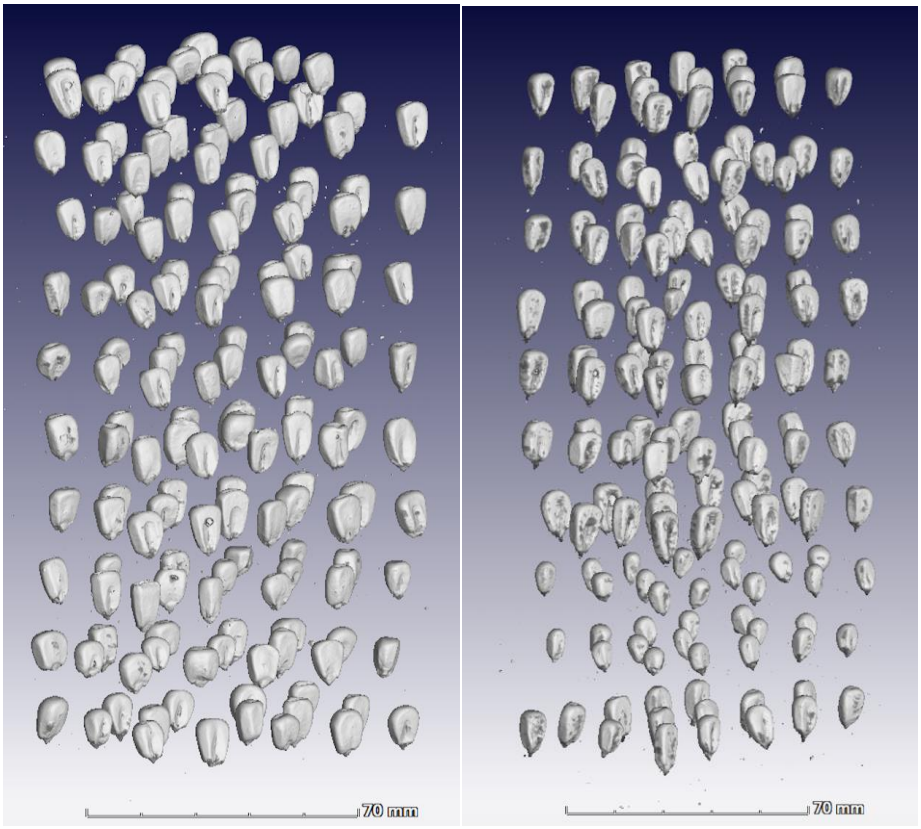
discs (Figure. 3.3). The ten discs were subsequently stacked on top of each other (150 kernels) and secured with a thin wooden rod to prevent any movement during X-ray acquisition. The procedure was then repeated for the hard maize samples.

All samples were scanned at a resolution of 140 µm with a constant of 60 kW voltage and 240 mA current. Acquisition per image was set at a constant 500 ms.

Figure 3.3. Digital images illustrating sample preparation for X-ray analysis.

3.3.4.4.1 Image processing and analysis

The acquired 2-D X-ray images were rendered into 3-D volumes, using the integrated Phoenix Datos acquisition and reconstruction software (General Electric Sensing and Inspection Technologies / Phoenix X-ray, Wunstorff, Germany). Figure 3.4 indicates 3-D images of the stacks of both hard (a) and soft (b) kernels. The process of reconstruction comprises of filtered back-projection algorithms where the grey values in a rendered CT image represent the attenuation in each pixel (Singhal *et al.*, 2013) (Figure. 3.4). The 3-D images were further analyzed with VG Studio Max 2.26 (Volume Graphics, Heidelberg, Germany).



a b

Figure 3.4. 3-D XCT images of two stacks of 10 discs, containing 15 kernels per disc and with the mounting material removed. a = hard genotypes and b = soft genotypes.

3.3.4.4.2 Volume analysis

Each maize kernel was analyzed independently as sub-volume extraction was possible. Volume analysis per kernel was thus performed. The volume of hard endosperm in relation to total endosperm was accurately (+/- 0.01g) determined.

Entire kernel volume (EKV) and the volumes of the two endosperm types, i.e. vitreous (VEV) and floury endosperm (FEV), was determined using the automated Region growing tool, in combination with the Volume analyzer function of VG Studio Max 2.26. The vitreous endosperm type volume (VEV) was then expressed as a percentage of the total volume of endosperm (EKV) per kernel.

3.4 Statistical analyses

Seventy-eight samples of the original 90 samples of known origin and cultivation method were analyzed to determine the effect of colour, cultivation method and climatic conditions on maize hardness. These samples were divided into three environmental categories of climatic classification of production according to the Köppen-Geiger system (Peel *et al*., 2007). Appendix 2 illustrates South African climatic conditions according to the Köppen-Geiger climate classification system (Peel *et al*., 2007):

- Cold semi arid
- Cold desert
- Humid subtropical

and two different cultivation methods:

- Irrigated
- Dry land

After investigation of a factorial analysis of variance (ANOVA) approach to determine possible interactions of relationships between maize colour and cultivation/climatic conditions in relation to NIR hardness index and PSI sieve values of samples, the data were analyzed with one-way analysis of variance ANOVA, as interactions could not be analyzed due to the low population of some of the interactions. Homogeneity of variance was then tested with Levene's test.

Mean differences for maize hardness determining methodologies (PSI, RVA, NIR at 2230 nm and XCT) of all 90 maize samples (Trial 1) and the 20 selected maize samples (Trial 2) were evaluated by one-way ANOVA using STATISTICA version 13 (StatSoft, Inc., Tulsa, USA) to determine possible relationships between methodologies. Pearson (r) correlations and correlation coefficients (r^2) were used to explain variation. Spearman's rank correlation coefficients were further used to test the strength of the relationships between pairs of the maize hardness testing methodology in a bivariate fashion to eliminate possible non-normal distribution.

As the maize hardness measurement data collected were not of the same units, data were subsequently sorted and ranked to perform Intraclass Correlation Agreement and Consistency (ICC); Müller and Büttner, 1994) as well as Bland and Altman (Bland and Altman, 1986) analyses in order to assess agreement between the methods of

measurement.

Significance was declared at $P \leq 0.05$ and tendencies at $P \leq 0.10$.

3.5 Results and discussion

The distribution of starch content (%) of all 90 maize samples is presented in Figure 3.5. The starch content (g/kg DM) of the 90 collected maize samples varied between 613.8 and 724.2 g/kg with a mean of 686.0 g/kg (Figure 3.5).

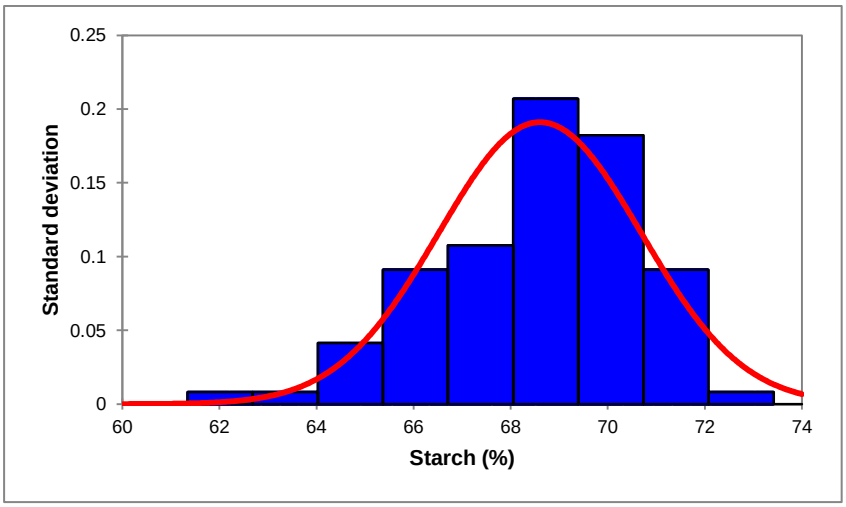


Figure 3.5. The distribution of starch content (%) of maize samples (n = 90).

The starch content of the 10 selected soft and 10 selected hard maize samples varied between 650.6 and 719.1 g/kg for the soft and between 613.8 and 690.4 g/kg for the hard samples. Mean starch content values of 695.1 and 669.5 g/kg were observed for the 10 soft and 10 hard samples, respectively and the difference was significant (P = 0.015, SEM = 6.17). Maize starch content in literature ranges from 650 to 760 g/kg DM (Kereliuk and Sosulski, 1996; National Research Council, 2001; McDonald *et al.*, 2002). Apart from differences in amylose and amylopectin content, Rooney and Pflugfelder (1986) reported no differences in starch content between maize of different hardness classes. In contrast, Blandino *et al*. (2010) proposed that starch content, moisture and fibre are all related to maize hardness. In support of the latter authors, the starch content of the 90 maize samples in this study indicated a higher starch

content for softer maize compared to that of harder maize samples when maize hardness was determined by NIR.

With respect to colour, no relationship in hardness index (as determined by NIR) was found between white and yellow maize genotypes with one-way ANOVA analysis. This non-significance was supported by Levene's test for homogeneity of variance. A coefficient of variation (CV) of 3.2% for yellow and 9.4% for white maize respectively nevertheless indicated a higher spread of hardness index data (NIR) for white compared to yellow maize. This is possibly due to the relative small amount of white maize samples in the population (8 of the 78 samples were white maize).

Least significant difference test *P*-values following ANOVA for maize hardness as variable compared to the climatic conditions where maize was produced are shown in Table 3.2. The NIR mean hardness indexes for the different climatic conditions of maize production were 7.29, 6.39 and 5.52 for cold arid, cold desert and humid subtropical, respectively. Despite the numerical differences and irrespective of cultivation method and colour in this study, maize was only significantly ($P \leq 0.01$) softer when cultivated in a humid subtropical climate compared to a cold desert or cold semi arid climates (Table 3.2). A tendency toward softer maize produced in cold desert climate compared to a cold semi arid climate was nevertheless observed (Table 3.2). All other climatic conditions of maize cultivation did not have an effect on maize hardness indexes (Table 3.2).

Table 3.2. Significant difference matrix of maize hardness (NIR hardness indexes) relationship with climatic conditions where maize was produced.

	Cold semi arid	Cold desert	Humid subtropical
Cold semi arid		0.063**	0.001*
Cold desert	0.063**		0.150
Humid subtropical	0.001*	0.150	

*$P < 0.01$
**$P < 0.1$

No effect on maize hardness index (NIR) was observed between dry land and irrigated cultivation practices when comparing cultivation practices by either ANOVA or Levene's test for homogeneity of variance. However, a CV difference of 7% and 3% for irrigation and dry land, respectively, indicate a higher spread of hardness index

data (NIR) for irrigation compared to dry land cultivated maize. This could also be explained by the relative small amount of irrigated samples in the population (16 of the 78 samples were produced by irrigation) compared to dry land.

Similar results were obtained when a PSI sieve test was used as maize hardness determination and subjected to the same statistical analysis as with NIR index values.

3.5.1 Trial 1

A NIR at a single absorbance of 2230 nm was used to produce hardness index values for the 90 maize samples and values ranged from 0 to 26 (Appendix 1). Guelpa (2015) reported maize hardness index values ranging from 0 to 15. Downey *et al.* (1986) reported a wheat hardness index range of 0 to 10 with the use of the same log equation that was used in the current study and that of Guelpa (2015). It would thus appear that maize hardness index values generated by NIR scanning in this trial were even more diverse in regards to vitreousness than that of Guelpa (2015) and (Downey *et al*., 1986). This suggests that the samples used in this study represent a diverse population regarding maize vitreousness.

The CV for NIR (47%) indicates greater variability than for PSI (8%) and thus indicates a wider normal data distribution for NIR at 2230 nm absorbance hardness results than that of PSI (Table 3.3).

Table 3.3. Descriptive statistics dialog.

Variable	N	Mean	Median	Minimum	Maximum	Lower Quartile	Upper Quartile	Standard Deviantion	CV
NIR	90	7.03	6.83	1.59	25.73	5.44	7.78	3.32	0.47
PSI	90	61.42	61.29	50.73	86.73	59.31	63.24	4.78	0.08

NIR = Hardness index with NIR at 2230 nm
PSI = Sieve at 106 µm

A strong correlation existed between the hardness index values calculated from NIR at 2230 nm absorbance and the PSI sieve test. A correlation coefficient (r^2) of 0.7437 ($P \leq 0.01$) was recorded. Spearman's rank order correlation further confirmed the

significant relationship (0.68 at P ≤ 0.01). A regression equation was fitted to best indicate the relationship between NIR and PSI sieve (Figure 3.6) methods:

$$\mathbf{y = 52.68 + 1.24x}$$

where y = PSI (% hard endosperm)

x = NIR hardness index (at 2230 nm)

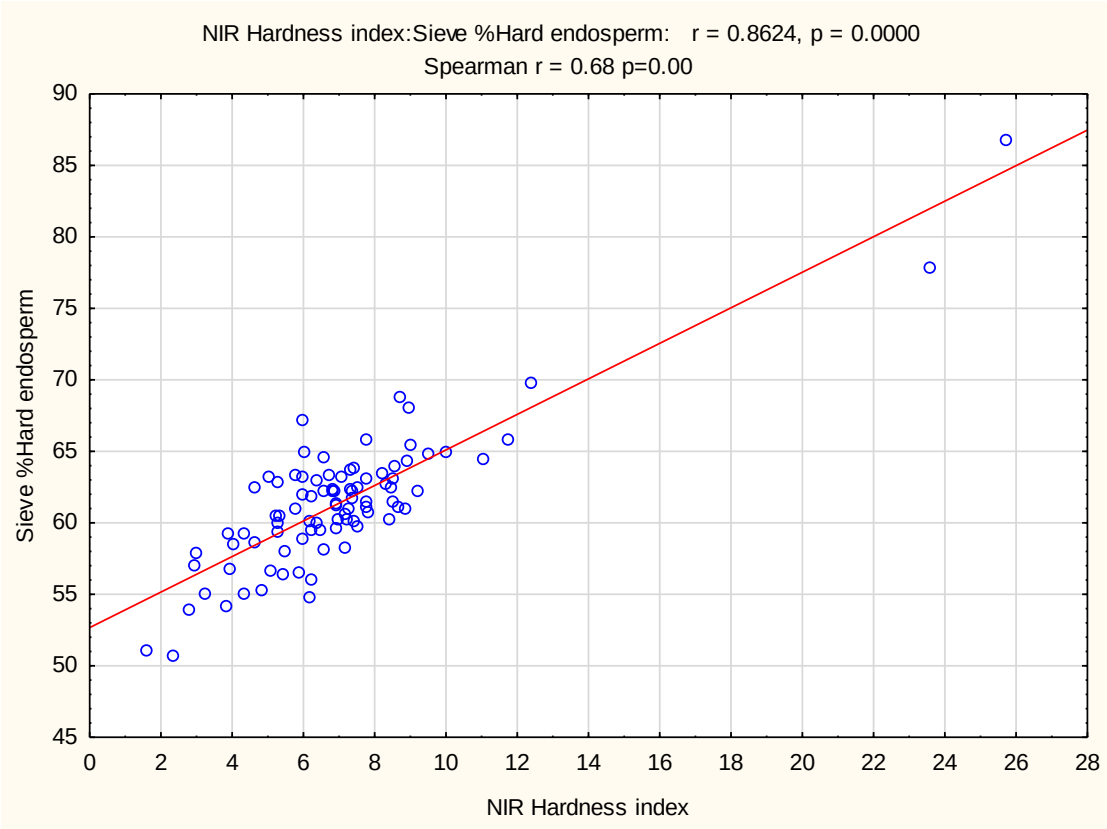


Figure 3.6. Scatterplot of NIR against PSI sieve.

The regression equation suggests that PSI measures maize hardness at a constant of 1.24 lower than the NIR.

To compare different methods of measurement, intraclass correlation (ICC) and Bland and Altman analyses were conducted. Due to differences in measurement units, the data were ranked and then sorted prior to analysis. The ICC analysis confirms a high degree of agreement (0.69) and consistency (0.68) between the relevant measuring methods. A high degree of confidence is supported by positive confidence intervals of consistency and agreement found with ICC analysis (Figure 3.7). In general maize

hardness determination as measured with PSI predicts the hardness of low vitreous maize higher than NIR. In contrast PSI determines hardness of high vitreous maize lower than NIR (Figure 3.7).

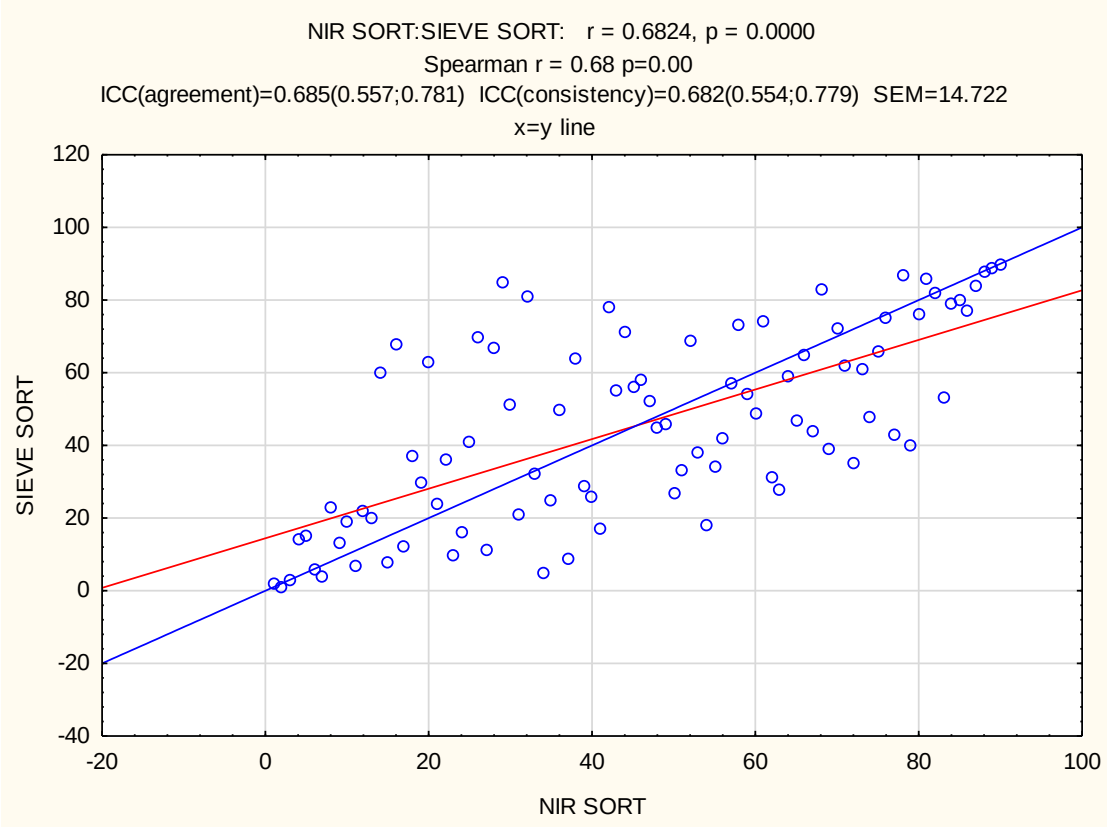


Figure 3.7. ICC scatterplot of sorted NIR against sorted PSI sieve.

Bland and Altman plots for maize hardness determining methods (PSI and NIR) are shown in Figure 3.8. Mean differences between NIR and PSI are 0, thus indicating a high degree of accuracy between the two methods of hardness determination. Figure 3.8 furthermore indicates a high degree of accurateness with extremely hard and soft maize (as measured with average sorted NIR and PSI). Despite ICC accuracy and agreement confidence (Figure 3.7), more variation within the middle of the range of average sorted data between the 2 methods is evident (Figure 3.8).

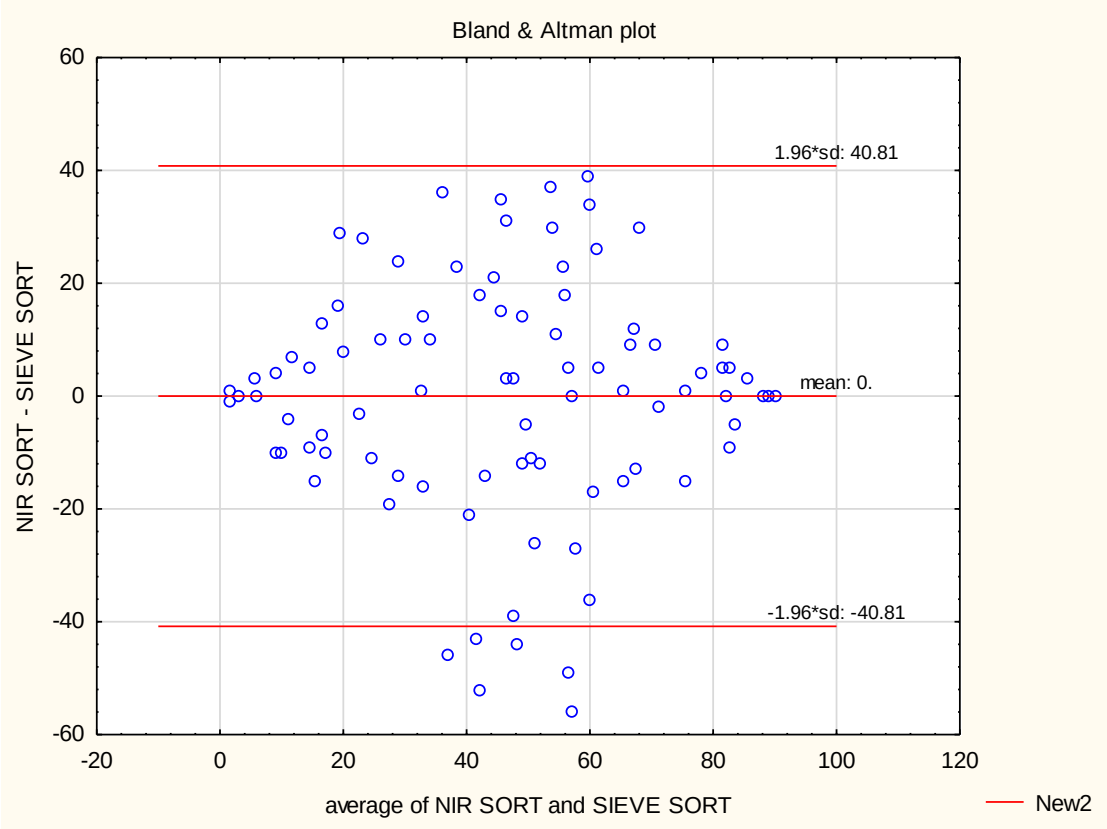


Figure 3.8 Bland and Altman plot of sorted NIR against sorted PSI sieve.

This study thus supports the proposal of Downey *et al*. (1986) that NIR at a single absorbance of 2230 nm is effective to determine maize hardness. The results of Trial 1 are further in accordance to findings reported by various other authors (Abdelrahman and Hoseney, 1984; Fox and Manley, 2009; Hoffman *et al.,* 2009; Guelpa, 2015) when evaluating various methods to determine maize hardness. Manley (2014) concluded in a NIR application review article that it has been shown that maize hardness can be accurately determined by NIR spectroscopy. Both tests required kernel destruction, but the feed industry is not kernel destructive sensitive. Accurate intact whole maize kernel NIR calibrations for vitreousness are possible. It could therefore be concluded from Trial 1 that both maize hardness methodologies of a single sieve through 106 µm mesh screen and hardness index calculated from a NIR single absorbance of 2230 nm are equally consistent and accurate to determine maize hardness. Results of Trial 1, in accordance with results of Corona *et al*. (2006) and Guelpa (2015), indicate that a V:F of < 1 (determined by PSI) and/or a NIR hardness index < 7 for milled maize, indicate low vitreousness.

3.5.2 Trial 2

An extensive review of various methodologies to determine maize hardness and the effect thereof on milling quality has been published by Fox and Manley (2009). Most of the research has nevertheless been done with respect to food science (Wehling *et al*., 1996) and not for application to the animal feed industry. As discussed earlier, the requirements for the different industries differ substantially. Reported methods that require kernel destruction, to accurately estimate maize hardness include PSI, Stenvert, Density, TADD (Tangential Abrasion Dehulling Device), RVA, Roff Milling Index and compression analysis (Fox and Manley, 2009). The Roff Milling Index has nevertheless been shown by Burden (2010) not to be an accurate indictor of ruminal starch degradability in dairy cows. Non-destructive methods to determine maize hardness include NIR and X-ray micro-computed tomography (XCT) (Guelpa *et al*., 2015ab). However, maize hardness determination techniques for application in the animal feed industry are not kernel destruction sensitive. Almost all maize used in the animal feed industry will be processed; therefore, there is no requirement to measure only intact kernels.

Three-D single maize kernel XCT images of the hardest vs. the softest maize samples analyzed are presented in Figures 3.9 and 3.10 respectively.

Soft endosperm is shown as yellow areas, while hard endosperm is indicated by red. The germ is represented by blue. From all angles (top, side and front), more soft endosperm (yellow) vs. hard endosperm (red) is evident in Figure 3.10 (soft maize) when compared to Figure 3.9 (hard maize).

According to the literature, XCT was shown to be accurate to determine maize hardness (Fox and Manley, 2009; Gustin *et al.,* 2013; Singhal *et al*., 2013; Guelpa, 2015; Guelpa *et al*., 2015b). The uniformity of results indicates that XCT could be used effectively to determine maize hardness. Due to the cost and difficulty to perform XCT, it cannot currently be recommended as a routine hardness test in the animal feed industry. However, due to the accuracy and consistency of results, XCT was selected in this study as a reference method against which other methods were tested.

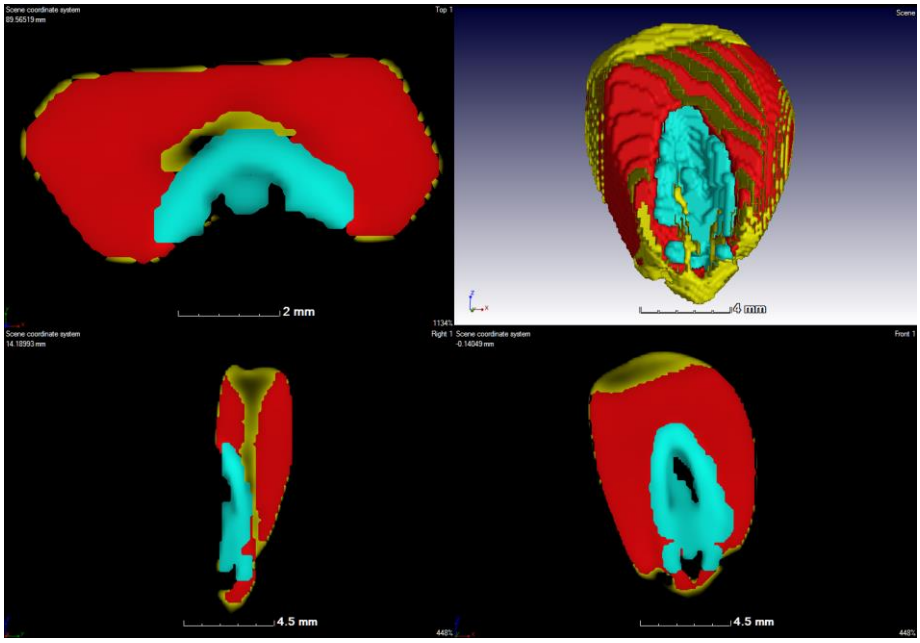


Figure 3.9. X-ray image of a hard maize kernel.

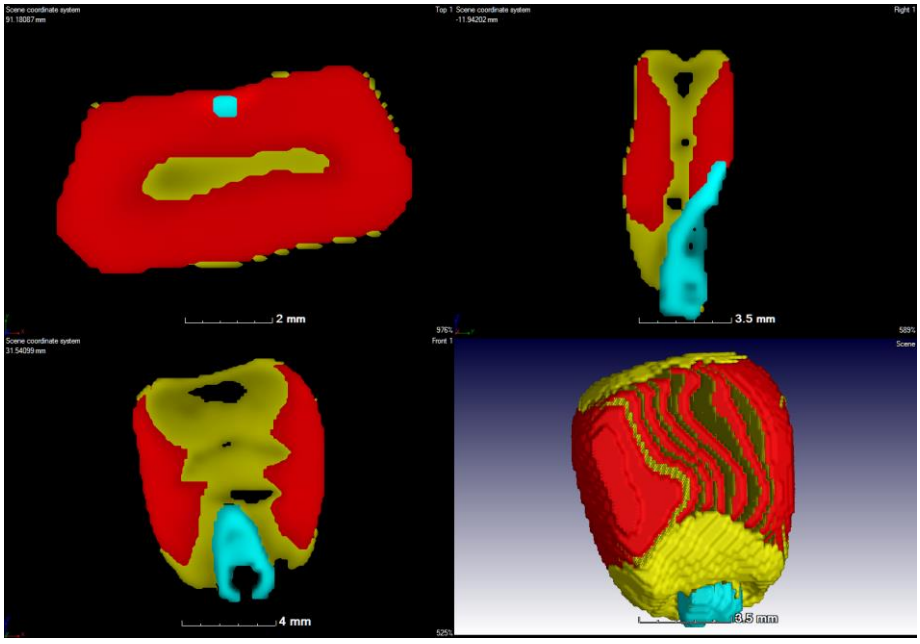


Figure 3.10. X-ray image of a soft maize kernel.

Result matrixes of correlation coefficient (r^2) and Spearman's rank order correlation coefficient of various hardness tests are presented in Tables 3.4 and 3.5 respectively.

Table 3.4. Correlation coefficient (r^2) matrix for various hardness tests (n = 20).

	PSI	NIR	X-ray	RVAPV	RVAHS	RVAFV	RVASV	RVAPT
PSI	1.000	0.917*	0.742*	-0.638*	-0.028	-0.001	0.001	0.724*
NIR		1.000	0.856*	-0.669*	-0.064	-0.000	0.011***	0.863*
X-ray			1.000	-0.473*	-0.022	0.000	0.005	0.797*
RVAPV				1.000	0.411*	0.020	-0.018	-0.522*
RVAHS					1.000	0.101	-0.011	0.081
RVAFV						1.000	0.825*	0.005
RVASV							1.000	0.002**
RVAPT								1.000

PSI = sieve at 106 µm
NIR = NIR at 2230 nm
X-ray = X-ray µCT
RVAPV = RVA (Peak viscosity)
RVAHS = RVA (Holding strength)
RVAFV = RVA (Final viscosity)
RVASV = RVA (Setback viscosity)
RVAPT = RVA (Peak time)
*: $P < 0.01$
**: $P < 0.05$
***: $P < 0.1$

As would be expected (due to selection), and in agreement to results of Trial 1, results from Trial 2 (Table 3.4) confirm the highly significant relationship between the PSI sieve test and NIR at a $r^2 = 0.917$ ($P \leq 0.01$). In concurrence, Spearman's rank order was 0.94 ($P \leq 0.01$) (Table 3.5). In agreement, Pomeranz *et al*. (1984) found similar results when comparing three methods (breakage susceptibility, NIR, and average particle size of ground material) to determine hardness of four types of maize. Pomeranz *et al*. (1984) concluded that all three investigated methodologies are equally acceptable to determine maize hardness.

Both PSI and NIR at $r^2 = 0.742$ ($P \leq 0.01$) and $r^2 = 0.856$ ($P \leq 0.01$) respectively correlated significantly with XCT method as reference technique for the determination of maize hardness (Table 3.4). These results were confirmed with a Spearman's rank order correlation of 0.76 ($P \leq 0.01$) and 0.82 ($P \leq 0.01$) respectively for PSI and NIR against XCT (Table 3.5).

Regression analysis of the selected samples indicated similar relationships between PSI and NIR as in Trial 1:

$$y = 52.24 + 1.28x$$

where y = PSI (%hard endosperm)

x = NIR hardness index (at 2230 nm)

Results from Trial 2 are similar and confirm that of Trial 1 and it could therefore be concluded that either of the two methodologies is equally accurate and useful to determine maize vitreousness. These results provide evidence that the selection of the samples for Trial 2 did not have an affect on accuracy of methods compared to Trial 1.

Regression equations were fitted to the data between XCT and NIR and PSI respectively:

$$y = -37.84 + 0.65x$$

where y = NIR hardness index (at 2230 nm)

x = XCT

$$y = 5.64 + 0.81x$$

where y = PSI sieve (106 µm)

x = XCT

These regression equations suggest that both the NIR and PSI sieve methodologies predict maize hardness lower than XCT and is supported by ICC and Bland an Altman analysis.

An ICC scatterplot analysis of PSI sieve against XCT (as reference) is presented in Figure 3.11 and indicates a high degree of agreement (0.62) and consistency (0.86) between the relevant measuring methods. A relatively high confidence interval for

consistency is supported by the positive confidence level (Figure 3.11). Despite the high degree of consistency, the analysis determined that maize hardness determination with PSI sieve, irrespective of hardness, is consistently lower than with X-ray µCT (Figure 3.11). The Bland and Altman plot analysis shown in Figure 3.12 confirm this relationship.

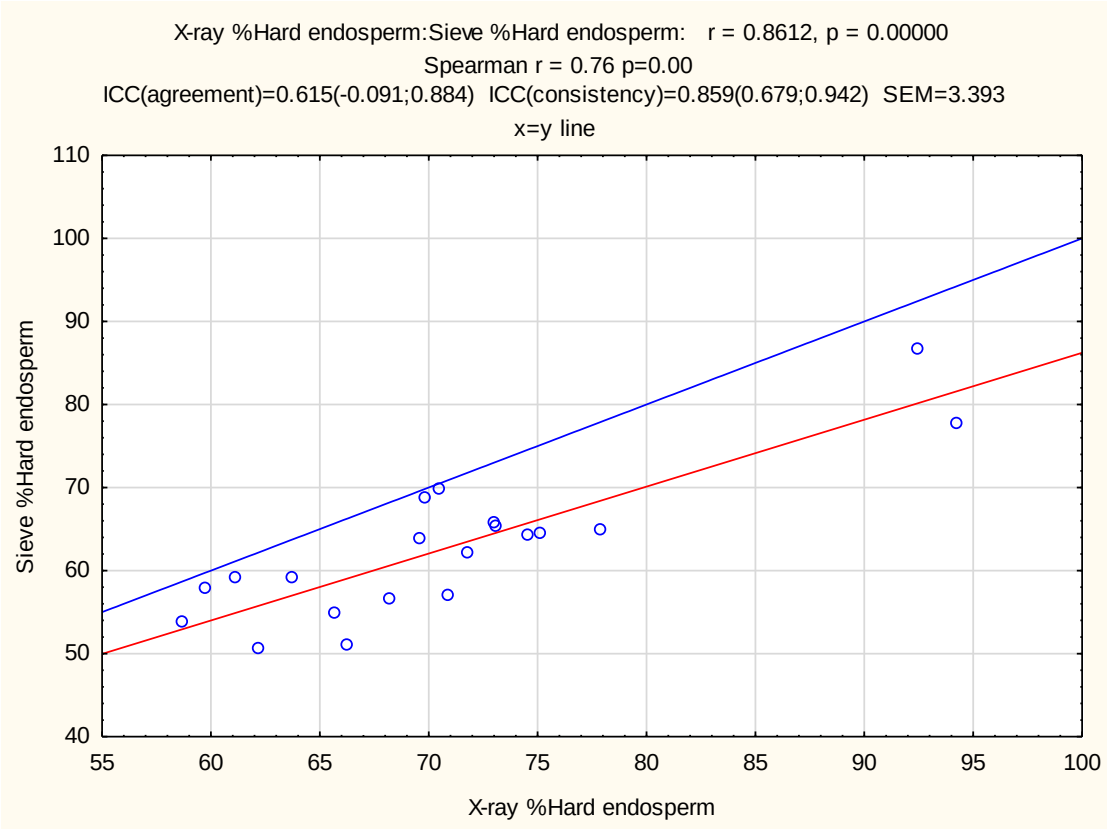


Figure 3.11. ICC scatterplot of PSI sieve against X-ray XCT.

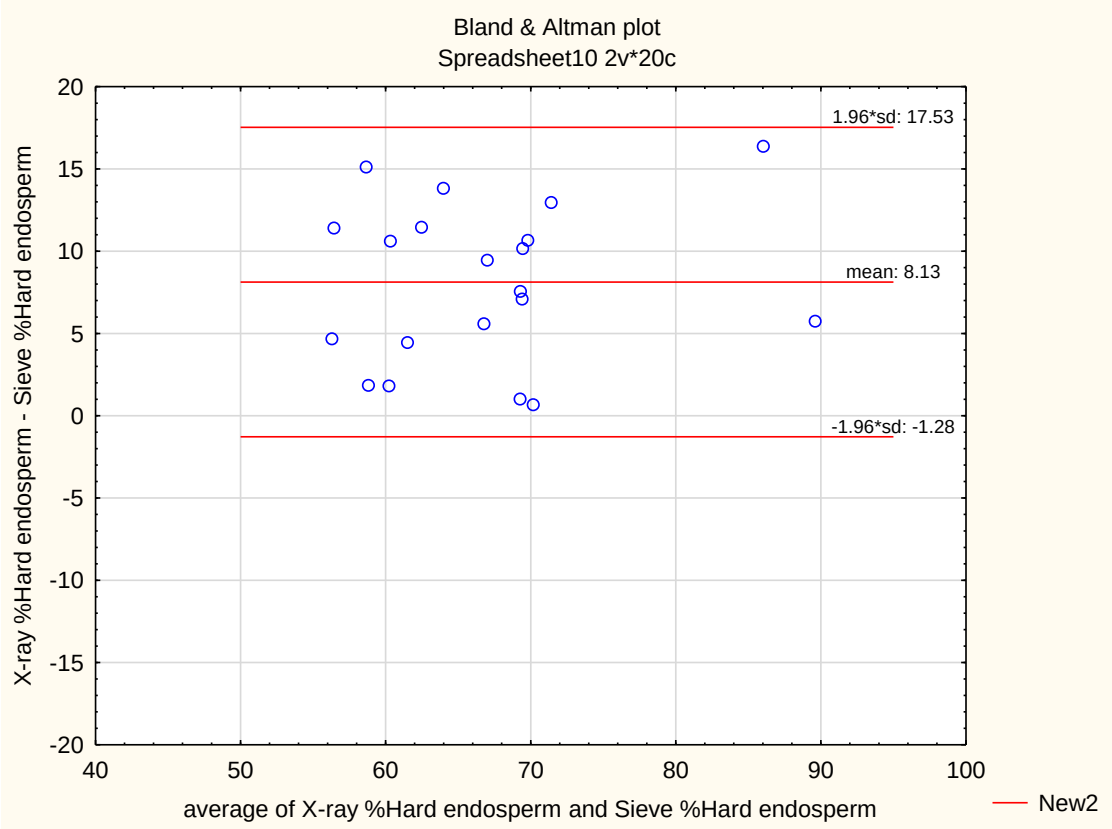


Figure 3.12. Bland and Altman plot of PSI sieve against X-ray XCT.

The RVAPV (peak viscosity) is described as the process of gelatinization and occurs at the equilibrium point between swelling and polymer leaching (which cause viscosity to increase) and rupture and polymer alignment (which cause viscosity to decline) when a grain sample is heated (Figure 3.2). The corresponding time required for a sample subjected to rheological analysis to reach peak viscosity is referred to as the RVAPT (peak time) (Figure 3.2). When comparing RVAPV (peak viscosity) maize hardness data in this study, a significant negative relationship (r^2) with PSI, NIR and XCT results were recorded (Table 3.4). Spearman's rank order correlation revealed similar results for RVAPV vs. XCT that differed significantly at -0.48 ($P \leq 0.05$) (Table 3.5). This negative relationship is in accordance to results of other authors (Yamin *et al*., 1999; Seetharaman *et al.,* 2001; Ji *et al*., 2003; Sandhu and Singh, 2007; Guelpa 2015).

Table 3.5. Spearman's rank correlation coefficient matrix for various hardness tests (n = 20).

	PSI	NIR	X-ray	RVAPV	RVAHS	RVAFV	RVASV	RVAPT
PSI	1.00	0.94*	0.76*	-0.67*	-0.13	0.11	0.23	0.83*
NIR		1.00	0.82*	-0.68*	-0.19	0.05	0.18	0.83*
X-ray			1.00	-0.48**	-0.03	0.19	0.31	0.82*
RVAPV				1.00	0.7*	0.13	-0.1	-0.55*
RVAHS					1.00	0.54*	0.3	0.05
RVAFV						1.00	0.94*	0.32
RVASV							1.00	0.42***
RVAPT								1.00

PSI = sieve at 106 µm
NIR = NIR at 2230 nm
X-ray = X-ray µCT
RVAPV = RVA (Peak viscosity)
RVAHS = RVA (Holding strength)
RVAFV = RVA (Final viscosity)
RVASV = RVA (Setback viscosity)
RVAPT = RVA (Peak time)
*: $P < 0.01$
**: $P < 0.05$
***: $P < 0.1$

In contrast to the negative correlation of RVAPV with other hardness parameters, results of this study found a strong positive relationship (r^2) between RVAPT and PSI, NIR and XCT (Tables 3.4 and 3.5). A significant ($P \leq 0.01$) relationship between NIR and RVAPT was established ($r^2 = 0.86$). With a correlation coefficient of 0.83 ($P \leq 0.01$), the Spearman rank correlation supports this data (Table 3.5). A very strong negative relationship ($r^2 = -0.52$; $P \leq 0.01$) was observed between RVAPV an RVAPT (Table 3.4). Table 3.5 further emphasizes the strong negative relationship by means of a Spearman's correlation coefficient of -0.55 ($P \leq 0.01$).

Regression analysis of RVAPV and RVAPT against XCT revealed:

$$\mathbf{y = 10541.67 - 82.5x}$$

where y = RVAPV

x = XCT

and

$$y = 58.83 + 3.59x$$

where y = RVAPT

x = XCT

These regression equations support the significant negative RVAPT and the strong positive relationship of RVAPV against XCT, thereby confirming the accurateness of rheological analysis to determine maize hardness.

The results of the current study support the theory that hard kernels show a more prominent protein-starch matrix compared to soft kernels and also require more time to gelatinize (Almeida-Dominguez *et al.*, 1997). The thicker protein matrix of vitreous endosperm thus forms a barrier that slows hydration (Wang and Eckhoff, 2000) and gelatinization (Narváez-González *et al.*, 2006).

It could thus be concluded that both RVAPV (negative correlation) and RVAPT (positive correlation) could be used as accurate methodologies to determine maize hardness. This result is in support of various other authors that have shown that the RVA can be used to quantify maize hardness (Yamin *et al*., 1999; Seetharaman *et al.,* 2001; Ji *et al*., 2003; Sandhu and Singh, 2007). This method nevertheless requires specialized equipment, lacks simplicity, and is costly and time consuming. Despite the accuracy, the use of RVA is thus not suited for routine maize hardness determination within the animal feed industry.

All other RVA parameters (RVAHS, RVAFV, RVASV) were not significantly related (Spearman and r^2) to PSI, NIR, XCT, RVAPV and RVAPT approaches (Tables 3.5 and 3.4). RVAHS, RVAFV or RVASV could therefor not be used to describe XCT. According to the current study, these RVA parameters could consequently not be used as a method to accurately describe maize vitreousness. Irrespective of the accuracy, the rheological data is also a difficult and time consuming to process.

3.6 Conclusion

Although, this study showed that RVAPV and RVAPT rheological analysis are accurate options to determine maize hardness compared to XCT, they lacked simplicity, speed or low cost as required by the animal feed industry for routine maize quality

determination. In contrast, this study (both Trials 1 and 2) showed that compared to XCT, both PSI through a single 106 µm mesh screen, as well as the use of a hardness index calculated from a single absorbance NIR at 2230 nm fulfilled the requirements of simplicity, accuracy, speed and low cost to determine maize hardness. It could further be concluded from results of this study that a V:F ratio of < 1 and/or NIR hardness indexes < 7 for milled maize indicate low vitreousness. Infinite sampling and analysis are also possible with both these methods. Furthermore, NIR technology is already extensively used throughout the animal feed industry. As results of the current study only indicated relative maize hardness index differences, it is thus recommended that accurate maize hardness NIR calibrations be developed to allow accurate reliable predictions. Break-even analyses techniques should be used to determine the impact of the cost of maize with different vitreousness compared to the relative animal production potential thereof. It was shown in the current study that all the animal feed industry's requirements to determine maize vitreousness could be met with the use of NIR technology at a single 2230 nm absorbance. The use of NIR scanning at a single absorbance of 2230 nm to determine maize vitreousness in an effort to predict the rate of ruminal starch disappearance (k_d) needs to be investigated. Simple, accurate, quick and inexpensive maize k_d determination with the use of a NIR in order to facilitate accurate formulation is required by the animal feed industry.

Results of this study therefore warrant further research to:

- Determine the possibility to change ruminal fermentation kinetics of maize of various vitreousness.
- Determine the relationship between the rate of ruminal starch disappearance (k_d) between batches of maize that vary in terms of vitreousness.
- Determine the relationship between NIR scanning and *in vitro* starch disappearance of maize that vary in vitreousness.

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CHAPTER 4

An evaluation of Near-infrared spectroscopy to estimate the rate and extent of starch degradation in maize of different vitreousness.

4.1. Abstract

Maize (Zea mays L) is a primary source of energy in diets of high producing ruminant animals. Thus, optimal maize utilization is fundamental in improving efficiency and animal production. Modern mechanistic dynamic models require accurate ruminal kinetic estimates to ensure accurate formulation predictions. In grain, the ratio of vitreous to floury endosperm determines vitreousness. Low vitreous maize contains less endosperm that is composed of densely packed starch granules embedded within a complex protein matrix, but contains more floury and loosely packed starch granules. Maize vitreousness is negatively correlated with ruminal starch fermentation. Near-infrared spectroscopy (NIR) technology at a single absorbance can be effectively used to predict vitreousness of milled maize. The objective of this study was therefore to determine the differences between milled (1 mm) maize of different vitrousness in terms of ruminal starch disappearance kinetics. A secondary objective was to determine if NIR hardness index values could be used to predict the extent and rate of ruminal degradation of maize starch. Six maize samples of decreasing vitreousness were selected from ninety samples with known vitreousness. The selected samples were incubated in vitro for 0, 3, 6, 12, 24 and 48 h whereafter ruminal starch disappearance parameters were determined by a first order model. Predicted ruminal starch disappearance (PRD) and fractional rate of starch degradation (k_d) decreased significantly as maize vitreousness increased. Hardness indexes calculated from NIR analyses at a single absorbance of 2230 nm showed inverse linear and quadratic relationships for both k_d and PRD. Linear coefficients were r^2 = 0.819 for k_d and 0.946 for PRD. Quadratic responses showed adjusted r^2 to be 0.917 for k_d and 0.993 for PRD. It was concluded that NIR technology can be used to predict ruminal fractional rate and extent of starch disappearance.

4.2 Introduction

Starch is a major energy-yielding component of cereal grains, which are important diet components used for intensive milk and beef production (Joy *et al.*, 1997; Shabi *et al*., 1999; Blasel, *et al*., 2006). For this reason, the efficiency of starch digestion by ruminants is of major importance (Nocek and Tamminga, 1991). Variation in terms of ruminal starch degradability is well documented (Huntington, 1997; Mills *et al.,* 1999; Firkins *et al.,* 2001). Some of the variation can be explained by different grain types (Stock and Britton, 1993; Dunshea *et al.,* 2012ab), genotypes (Philippeau *et al*., 1997; Correa *et al*., 2002; Allen *et al*., 2008; Ngonyamo-Majee *et al*., 2008b; Lopes *et al*., 2009) and processing methods (Theurer, 1986; Rowe *et al*., 1999; Callison *et al*., 2001; Rémond *et al.,* 2004; Dehghan-banadaky *et al*., 2007; Gencoglu, *et al*., 2010; McCarthy, *et al*., 2013).

In grains, the higher the ratio of vitreous to floury endosperm ratio (V:F), the harder the kernel is (Ngonyamo-Majee *et al*., 2008ab). Harder, vitreous endosperm is composed of densely packed starch granules embedded within a complex protein matrix, whereas the softer, floury endosperm contains larger, loosely packed starch granules (Lee *et al.,* 2006). A negative effect on ruminant animal performance of high vs. low vitreous maize has also been well documented (Firkins *et al*., 2001; Ngonyamo-Majee *et al*., 2008a; Allen *et al*., 2008; Hoffman and Shaver, 2009). Increased kernel vitreousness reduced ruminal maize starch degradation *in situ* (Philippeau and Michalet-Doreau, 1997; Correa *et al*., 2002; Ngonyamo-Majee *et al*., 2008ab) while Taylor and Allen (2005) reported both lower ruminal and total tract starch digestibility with increased vitreousness. The latter authors also reported a significantly slower rate of degradability of 1.8%/h vs. 7.7%/h for high vitreous compared to low vitreous maize. The strong starch-protein matrix of high vitreous grain, limit rumen micro-organisms (RMO) access to kernel starch and is responsible for slower ruminal starch fermentation rates compared to low vitreous grain (Rooney and Pflugfelder, 1986; McAllister *et al*., 1993; Opatpatanaki *et al*., 1994).

Grain processing increases the availability of starch in floury endosperm more than starch in vitreous endosperm (Huntington, 1997). High amounts of amylopectin in the floury endosperm (Rooney and Pflugfelder, 1986) are completely disrupted when processed, releasing free starch granules. In contrast, there is little release of starch granules during processing for vitreous endosperm because the protein matrix is thicker and stronger (Watson and Ramstad, 1987; Corona *et al*. (2006). It is therefore

generally assumed that maize with a higher proportion of floury endosperm might have a higher starch digestibility and be more responsive to processing (Rowe *et al*., 1999).

Modern feed evaluation systems are changing from static empirical models towards mechanistic dynamic models (Jones *et al.,* 2016). Dynamic models of digestion are more accurate at predicting the nutrient supply to animals under a wide range of conditions, because they predict intake more accurately, and they can deal with more complex diets and their interactions (Herrero *et al.,* 2013; Tedeschi *et al.,* 2014). A mechanistic model of starch digestion has the potential to improve predictions of substrate supply with regard to the effect of the site of starch digestion on the profile of absorbed nutrients (Tedeschi *et al.,* 2005; Huhtanen & Sveinbjörnsson, 2006). To be successful, this approach requires reliable and accurate predictions of nutrient supply from the digestive tract including ruminal kinetic parameters of starch digestion (Huhtanen & Sveinbjörnsson, 2006; Sniffen and Ward, 2011). Therefore, the rate and extent of fermentation of dietary carbohydrates (especially starch) in the rumen are important parameters that determine nutrient supply to the animal (Hall, 2004). Dynamic models use mechanistic equations that predict a variable TDN and microbial protein yield based on variables such as fermentable structural and non-structural carbohydrate (NSC) intake, rates of fermentation, the availability of amino N, and pH (Sniffen *et al.,* 1992). According to Waldo and Smith (1972), the fractional digestion of a potentially digestible component in the rumen is indicated by the digestion rate (k_d) over the sum of digestion rate (k_d) and passage rate (k_p). Passage rate (k_p) is determined normally with rumen evacuation techniques or digesta markers and calculated as the flow of undigested residues from the rumen divided by the rumen volume of digesta (Van Soest, 1994; Firkins *et al.,* 1998). Typical maize passage rates of 3-4 %/h is reported by Sniffen *et al.* (1992) and 3-7 %/h by Van Soest (1994). Potential ruminal starch disappearance (PRD) in the rumen can therefore be calculated by (Waldo and Smith, 1972; Sniffen *et al.,* 1992):

$$\mathbf{PRD = k_d/(k_d+k_p)}$$

Fractional rate of starch degradation in the rumen (k_d) can be estimated using different methods including *in situ* and *in vitro* methods to determine starch disappearance and also including gas production methods (Huhtanen & Sveinbjörnsson, 2006). All the methods used to estimate *in vivo* ruminal starch digestibility and the rate of starch degradation have problems; only total tract starch digestibility can be measured with a small error (Huhtanen & Sveinbjörnsson, 2006). These methods are, despite differences in accuracy (Huhtanen & Sveinbjörnsson, 2006) also costly, time

consuming and impractical for rapid, regular industry use.

Near infrared (NIR) spectroscopy has been shown to be accurate to estimate maize hardness (Pomeranz *et al.,* 1984; Eyherabide, *et al.,* 1996; Wehling, *et al.,* 1996; Guelpa, 2015). A single wavelength of 2230 nm has further been shown to be accurate to predict hardness in milled maize (Guelpa, 2015) and wheat (Downey *et al*., 1986). This was confirmed with results in an earlier study (Chapter 3) of this dissertation. NIR technology has also been shown to be a valuable tool to predict unreached starch during storage, handling, and processing of ethanol from maize (Plumier *et al.,* 2013). It could therefore be advantageous to exploit NIR technology to predict the fractional rate of degradation (k_d) and potential ruminal starch disappearance (PRD) of maize.

The objective of this study was thus to determine:

- The differences in rate and extent of *in vitro* starch disappearance of maize of varying vitreousness.
- The usefulness of NIR scanning at a single absorbance of 2230 nm to predict the fractional rate of degradation (k_d) and extent (PRD) of maize starch.

4.3 Material and methods

In this study, ruminal rate and extent of starch degradation was determined by an *in vitro* starch disappearance method.

4.3.1 General

Six maize samples of varying vitreousness were selected from a set of ninety samples (1kg each) that were collected throughout South Africa, and including a few from Argentina and Ukraine. These samples originated from the same set as described in Chapter 3. Selection of the six samples for the current study was based on the ranking of vitreousness as determined by NIR at a single absorbance of 2230 nm in a previous study (Chapter 3). In the selection of the samples, the aim was to have a set of samples that represented vitreousnes from very soft to very hard. The NIR hardness index values of the selected six samples are presented in Table 4.1. Vitreousness increased arbitrarily from 1 to 6 where 6 was popcorn.

Table 4.1. The NIR hardness index values of six maize samples selected for the *in vitro* starch disappearance trial.

Vitreousness[1]	NIR (2230 nm)[2]
Sample	Hardness index
1	1.59
2	5.08
3	7.06
4	8.53
5	12.41
6	25.73

[1]The vitrousness number is an arbitrary value, where 1 = very soft and 6 = very hard.
[2]The NIR hardness index was calculated based on a log formula described in Chapter 3.

4.3.2 Sample preparation

Grain grind sizes of 1-6 mm and incubation periods of 6-12 hours are commonly used with *in vitro* digestibility studies (Taysom, 2013). Evaluating the effect of sample processing procedures on measurement of maize starch, Hall and Mertens (2008) recommend drying samples at cooler temperatures (55°C) in forced air ovens and grinding through a 1 mm screen with an abrasion mill to reduce variability and to achieve higher starch values with maize silage and grain (Hall and Mertens, 2008). Hall (2009) also suggests a 1 mm grind size to establish a standard procedure for starch analysis of purified substrates and flour in animal feeds. All samples were therefore milled through a standard laboratory mill (Scientec RSA Hammer mill Ser. Nr 372; Centrotec) to pass through a 1 mm screen and were subsequently stored in air tight honey jars while holding samples were vacuum sealed. After preparation, all samples were stored at -18°C to prevent pest infestation.

The DM content of all the maize samples was accurately determined after drying sub-samples at 105°C for 24 h in a forced air oven.

4.3.3 Rumen fluid collection

Fresh rumen fluid was collected prior to each *in vitro* run from two ruminally cannulated lactating Holstein dairy cows. Rumen fluid collection for this trial was consistently at 10h00 in the morning, exactly 3 hours after the morning feeding. A retention time of 3 hours was chosen to ensure rumen fill with fresh TMR and adequate RMO activity (Weimer, 2017). All rumen collections were done in accordance to the rumen extraction protocol of the University of Stellenbosch and the trial were approved by the Stellenbosch University's Animal Ethics Committee (reference: SU-ACUD16-00157). Cows were from the Welgevallen Experimental Farm's herd of the University of Stellenbosch, South Africa. The cows received a TMR *ad libitum* consisting of lucerne hay (310 g/kg DM) and wheat straw (18 g/kg DM) mixed with a commercial energy-protein-mineral-vitamin concentrate (619 g/kg DM) and molasses meal (53 g/kg DM). Water was used to balance the moisture content of the TMR to 550 g/kg. The TMR was fed *ad libitum* twice daily at 07h00 and 16h00.

Rumen fluid was collected from multiple areas in the rumen and immediately filtered through two layers of cheesecloth before being transported to the laboratory in prewarmed thermos flasks. The flasks were filled to the brim to keep the contents anaerobic. In the laboratory the content of each flask was subsequently strained through four layers of cheesecloth into prewarmed (39.6°C) glass beakers (2 L) and gassed continuously with a gentle stream of CO_2 until used.

In this study the rumen fluid of the two cows were pooled at equal amounts to create one single combined rumen fluid inoculant. The final combined content of each run was again gassed continuously with a gentle stream of CO_2 until used.

Pooled rumen fluid pH varied between 5.8 and 6.0 between runs. Incubations with rumen fluid from ruminally cannulated cows on different diets showed similar ranking orders of different starches with respect to rate and extent of degradation (Huhtanen and Sveinbjörnsson, 2006), therefore the rumen fluid used with *in vitro* incubation is not that dependent on a specific diet of the cow (Weimer, 2017). The observed rumen fluid pH variation between different animals and runs of collected fluid was very small and could be attributed to various factors, such as variation in feed and water intake, dominance and reproductive cycle on the day of collection (Weimer, 2017).

4.3.4 *In vitro* solutions

All *in vitro* samples were incubated in a buffered incubation medium containing a rumen fluid inoculum, as described by Goering and Van Soest (1970) and Van Soest and Robertson (1991). Rezasurin solution was prepared by dissolving 0.1g of rezasurin into 100 mL of distilled water (dH_2O), creating a 0.1% solution (Goering and Van Soest, 1970). The solution was subsequintly stored in a glass container at 4°C. The reducing solution was prepared in two separate flasks, A and B. The content of each was stirred and left until fully dissolved, followed by the gentle addition and mixing of the solution in flask B to that of flask A prior to mixing to the buffer solution. Subsequintly the reducing solution were mixed with the incubation medium immediately prior to onset of incubation. The final *in vitro* solution had a pH of 7.3. The various solutions used are presented in Table 4.2.

Table 4.2. Composition and mixing of the *in vitro* solutions (Goering and Van Soest, 1970).

Reagent	Quantity
1 L Buffer solution:	
Distilled water (dH_2O)	1000 mL
Ammonium bicarbonate (NH_4HCO_3)	4 g
Sodium bicarbonate ($NaHCO_3$)	35 g
1 L Macromineral solution:	
Distilled water (dH_2O)	1000 mL
Di-sodium hydrogen orthophosphate (Na_2HPO_4) (anhydrous)	5.7 g
Potassium dihydrogen orthophosphate (KH_2PO_4) (anhydrous)	6.2 g
Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)	0.6 g
100 mL Micromineral solution:	
Calcium chloride dihydrate ($CaCl_2.2H_2O$)	13.2 g
Manganese chloride tetrahydrate ($MnCl_2.4H_2O$)	10 g
Cobalt (II) chloride hexahydrate ($CoCl_2.6H_2O$)	1 g
Ferric chloride hexahydrate ($FeCl_3.6H_2O$)	8 g
3.7 L Incubation medium (80 samples):	
Distilled water (dH_2O)	1600 mL
Tryptose	8 g
Micromineral solution	400 µL
Macromineral solution	800 mL
Rezasurin	4 mL
160 mL Reducing solution (80 samples):	
Flask A:	
Distilled water (dH_2O)	80 mL
Cysteine hydrochloride ($C_3H_7NO_2$ HCl)	1 g
Potassium hydroxide (KOH) pellets	40
Flask B:	
Distilled water (dH_2O)	80 mL
Sodium sulphide nonahydrate (NaS)	1 g

4.3.5 *In vitro* starch disappearance

Starch degradation is commonly measured *in vitro* directly by measuring starch disappearance after incubation for various time intervals (Menke *et al.*, 1979). Most methods involve incubations of feed samples in buffered rumen liquor, more or less based on the original method of Tilley and Terry (1963) for predicting apparent digestibility. More recently Richards *et al.* (1995) described a method for *in vitro* starch

disappearance, which forms the basis for all *in vitro* starch disappearance study.

Amounts of 300 ± 10 µg of each prepared maize sample plus a 20 mm magnetic stirrer bar were placed into 250 mL Nalgene plastic bottles. Buffered rumen liquor inoculated medium was used for the *in vitro* incubations. A surgical syringe was used to add 40 mL of the buffered medium into each bottle. After the reagents had been added, gassed with CO_2 and the incubation buffer had been reduced, rubber stoppers were placed on the containers. The containers were subsequently transferred to the incubation chamber and placed on a magnetic stirrer plate. A timer (set at 15 minutes per hour) was used to control stirring time automatically. The temperature of the incubator chamber was maintained at a constant 39.6°C throughout the entire incubation period.

For the current *in vitro* starch disappearance trial samples were incubated for 0, 3, 6, 12, 24 and 48 h intervals. For the 0 h time point, the maize substrates were soaked in 50 mL of distilled water in 250 mL Nalgene plastic bottles for 1 h at room temperature (26°C) before analysing for starch. All other incubated samples were removed from the incubation chamber at the appropriate times and immediately placed on ice for 30 minutes to stop fermentation. Cooled samples were subsequently stored at 4 °C for starch analyses. Blank samples, without substrate, were analyzed for starch to correct for starch within the rumen fluid alone. Results from four runs were recorded.

4.3.6 Starch analysis

In the current study, starch analyses for both the substrate and the *in vitro* digesta residues were based on the method as described in by Hall (2009) in Chapter 3.

4.3.7 Estimation of kinetic coefficients

The Solver option in Microsoft Office Excel (2013) and a non-linear model that included a lag phase were used to calculate the kinetic coefficients from the *in vitro* apparent starch disappearance analyzed results. The first derivatives a, b, c (k_d) and L were determined with the fitment of the data to a first order non-linear model and was based on a modified version of that described by Ørskov and McDonald (1979).

$$Y = a + b\,(1 - e^{-c(t-L)})$$

where Y = starch disappearance at time t
a = dissolved starch
b = potential degradable starch
c = fractional rate of degradation (%/h)
t = incubation time (hours)
L = lag time (hours)

The predicted ruminal starch disappearance (PRD) was subsequently calculated (Batajoo and Shaver, 1998; Bal and Shaver, 2006) as:

$$PRD = a + b\,[k_d / (k_d + k_p)]$$

where PRD = predicted ruminal starch disappearance
a = starch that disappeared after soaking in dH_2O for 30 minutes
b = potentially degradable starch
k_d = fractional rate of degradation (%/h)
k_p = Passage rate (%/h)

Both the a and b fractions were not chemically defined, but mathematically determined. Passage rate (k_p) was assumed to be 0.07 %/h (Batajoo and Shaver, 1994; Batajoo and Shaver, 1998).

4.4 Statistical analysis

A non-linear first order model (modified from Ørskov and McDonald, 1979) was used to predict starch disappearance at time t, dissolvable starch (a), potential degradable starch (b), fractional rate of starch degradation (c) and lag time (L) with the Solver option in Microsoft Office Excel. The first deritives were then used in a secondary model (Batajoo and Shaver, 1998; Bal and Shaver, 2006) to determine predicted ruminal starch disappearance (PRD). All the kinetic coefficients were then subjected to a main effects ANOVA with the aid of Statistica, version 13 (Stat Soft, Inc., Tulsa, USA). Relationships between NIR and k_d and PRD were determined with regression analysis, whereafter one-way ANOVA with the aid of Statistica, version 13, was used

to determine significance between parameters. Significantly different means were separated with a Bonferroni test. Significance was declared at $P \leq 0.05$ and tendencies at $P \leq 0.10$.

4.5 Results and discussion

According to Taysom (2013) trying to mimic *in vivo* digestibility with *in vitro* techniques is neither the goal, nor a realistic expectation. Taysom (2013) suggested that *in vitro* analysis can only evaluate the potential and relative digestibility of a feedstuff.

In a study evaluating the effect of processing techniques on *in vitro* degradability of maize, Lee *et al.* (2002) used 0, 2, 6, 12, 24 and 48 hours incubation to determine the fractional rate of degradation. It is generally accepted in literature that 6 time points are required to determine fractional rates of degradation (Batajoo and Shaver, 1998; Bal and Shaver, 2006; Hall, 2017; Weimer, 2017).

The *in vitro* starch degradability of maize of different vitreousness is presented in Figure 4.1. It can be seen in Figure 4.1 that, in general, ruminal maize starch degradation of most types of maize used in this study commenced early and increased rapidly from 3 h, peaked at more or less 6 to 10 h and reached an asymptote between 24 and 30 h. These results are similar to gas production results reported by Hoffman *et al.* (2012), where they evaluated the rate of ruminal starch degradation of different grains and reported that degradation commenced and increased rapidly after 4 h of incubation and peaked after approximately 6 h. Thereafter, degradation declined to almost insignificant levels after 24 h of incubation (Hoffman *et al.*, 2012).

In maize, the protein is mainly in the form of zein, which is of a poor quality with low levels of lysine and tryptophan (Larson and Hoffman, 2008). These are storage proteins (Fox and Manley, 2009) and because of their insolubility in rumen fluid, they are poorly degraded in the rumen (Rowe *et al*., 1999). Ruminal degradation would be limited when high vitreous maize is fed due to maize starch granules which are surrounded by zein and thus being encapsulated in a rigid protein-starch matrix (Kotarski *et al*., 1992; Johnson *et al*., 1999; Gibbon *et al*., 2003).

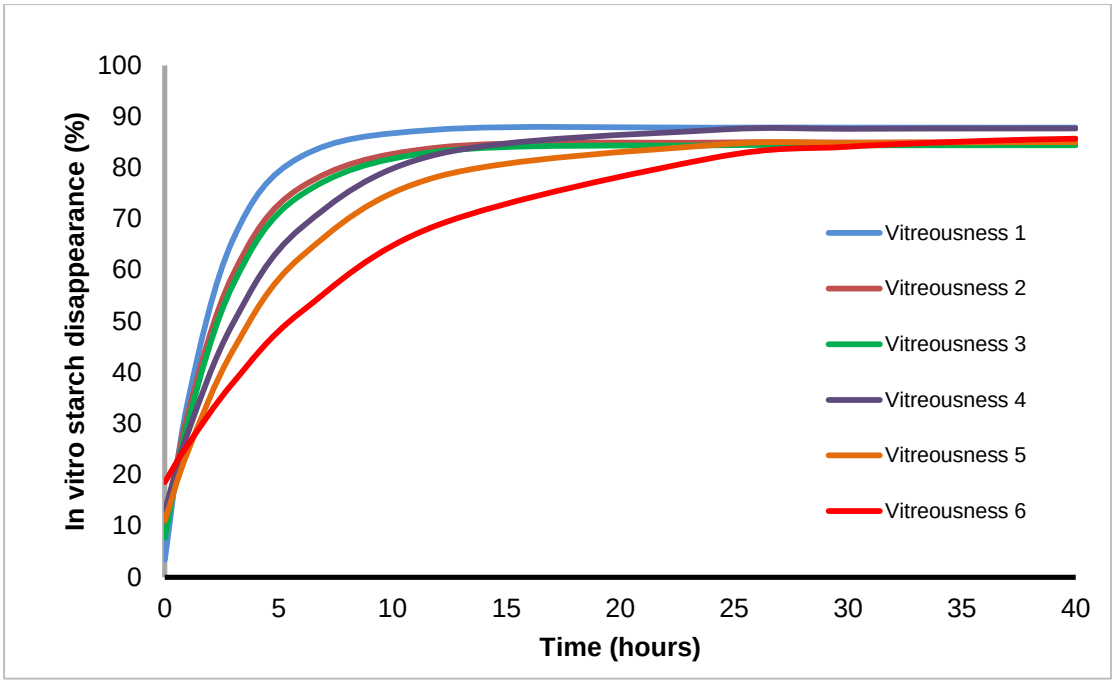

Figure 4.1 The effect of maize vitreousness on *in vitro* starch disappearance. Vitreousness values are aribtrary with 1 = very soft and 6 = very hard (popcorn).

Depending on type, extent of processing (high moisture, steam flaked, steam rolled, fineness of grind) and the genetic background, floury to vitreous (low to high prolamin), maize will have varying amounts of fermentable starch that disappear within two to three hours (Sniffen and Ward, 2011). In the current study all samples, except Sample 6 (popcorn), also showed a high degradation rate from 2 to 5 h of incubation (Figure 4.1), more so for the low vitreous maize. The fairly rapid degradation can be attributed to the fine grinding of the samples.

Results of the non-linear parameters and predicted ruminal starch disappearance are presented in Table 4.3.

The observed differences in disappearance rate between samples (Table 4.3) can be explained by the differences in vitreousness. As Sample 6 is extremely hard, the signifantly slower rate, especially during the first 5 h of incubation (Table 4.3), was to be expected. The negative impact of increased kernel vitreousness on ruminal maize starch degradation is well documented (Philippeau and Michalet-Doreau, 1997; Correa *et al*., 2002; Taylor and Allen, 2005; Ngonyamo-Majee *et al*., 2008ab). Taylor and Allen (2005) reported a significantly ($P \leq 0.05$) slower rate of degradation of 1.8 %/h vs. 7.7

%/h for high vitreous compared to low vitreous maize.

Table 4.3. The effect of maize vitreousness on *in vitro* non-linear parameters and predicted ruminal disappearance of starch.

Item	Vitreousness[1]						SEM	*P*
	1	2	3	4	5	6		
a, %[2]	20.2[a]	19.7[ab]	18.6[bc]	20.0[a]	17.6[c]	21.8[d]	0.453	<0.001
b, %	67.7[a]	65.2[ab]	65.8[ab]	67.7[a]	67.4[ab]	64.6[b]	0.618	<0.01
k_d,	0.452[a]	0.363[b]	0.346[b]	0.225[c]	0.200[c]	0.112[d]	0.010	<0.001
lag, h	0.49[a]	0.44[b]	0.45[b]	0.44[b]	0.47[ab]	0.44[b]	0.006	<0.001
PRD, %[3]	78.8[a]	74.4[b]	73.3[bc]	71.5[c]	67.5[d]	61.6[e]	0.504	<0.001

[a-d]Means in the same row with different superscripts differ ($P \leq 0.05$).
[1]Vitreousness was determined with the aid of a single spectrum NIR absorbance procedure and arbitrary numbers were assigned to indicate the vitreousness of six out of 90 maize samples that were selected to represent vitreousness over a spectrum of low (1) to high (6). The highest vitreous sample (6) was popcorn.
[2]Non-linear parameters a = starch that disappeared after soaking in distilled water for 30 minutes; b = potentially degradable starch; k_d = fractional rate of degradation; lag = time before fermentation commences.
[3]PRD = Predicted ruminal disappearance.

Although not investigating vitreousness, Seo *et al*. (2009) in contrast to Taylor and Allen (2005), with *in vitro* gas production techniques, reported an absolute higher k_d value of 18.25 %/h for maize. Based on a meta-analysis of several studies (Waldo *et al.,* 1972; Mertens, 1973; Mertens and Ely, 1979; Ewing and Johnson, 1989; Van Soest *et al.,* 1981; Krishnamoorthy *et al.,* 1983, Hoover, 1983; Smith *et al.,* 1972), Sniffen *et al.* (2014) reported even higher absolute maize k_d values ranging from 10-15 %/h, 15-20 %/h and 30-40 %/h for whole, coarsely rolled and finely rolled maize respectively. The observed absolute k_d values in this study decreased linearly from 45 %/h to 11.2 %/h as vitreousness increased. The relative high absolute k_d values found in the current study (Table 4.3) compared to some other authors could be attributed to the fine processing of the samples (1 mm screen) and the specific rumen fluid used. Extracted ruminal fluid pH variations of < 6 indicate that the RMO population in the donor cows were well adapted to high amounts of starch (Chen *et al*., 1995; Nocek, 1997; Rowe *et al*., 1999; Deckhardt *et al*., 2013) and is related to the 3 h retention time from feeding to rumen fluid extraction. Taysom (2013) also warns that *in vitro* analysis can only evaluate the potential and relative digestibility of a feedstuff. Despite the differences in absolute k_d values, results of the current study in support to that of literature, indicated a decrease in fractional rate of starch degradation as maize

vitreousness increased (Figure 4.1 and Table 4.3). With *in vitro* studies based on starch degradation McAllister *et al.* (1993) concluded that the protein-matrix is the major factor responsible for differences in ruminal degradation between maize and barley. Results of this study therefore supports the theory that the stronger starch protein matrix of higher vitreous maize limit the RMO access to kernel starch and is responsible for slower ruminal starch fermentation rates compared to low vitreous maize (Rooney and Pflugfelder, 1986; Opatpatanaki *et al*., 1994).

Despite the fact that some significant differences between the soluble/rapidly degradable fractions (a) of starch in the different vitreous maize samples were observed, no particular pattern could be established (Table 4.3). The observed differences are difficult to explain, but the relative higher standard error (SEM) suggests that the rapid degradable fraction (a) estimation might be relatively far from the population mean. Reported values for the a-fraction have also been highly variable, both between and within feeds (Nocek and Tamminga, 1991; Offner *et al.,* 2003). The a-fraction is typically assumed to have an infinite rate of degradation (Ørskov and McDonald, 1979) or an extremely fast rate such as 2.0 - 4.0 /h (Sniffen *et al.,* 1992). Although some of the differences between maize types regarding the a-fraction in the current study were significant ($P < 0.001$), the differences were small and probably of no biological significance. Higher vitreous maize have high levels of amylose whereas less amylose is present in low vitreous maize (Rooney and Pflugfelder, 1986). Amylopectin is soluble in water at room temperature, while amylose is not (Green *et al.,* 1975), therefore one explanation might be related to differences between maize samples regarding amylose:amylopectin ratios. However, in the current study, the a-fraction did not appear to change according to vitreousness, which does not support this hypothesis.

Obeserved lag times also did not show any particular pattern, but were lower than 0.50 h for all samples, indicating relative short lag times (Table 4.3).

Regarding predicted ruminal disappearance (PRD), a linear decrease (ranging from 78.8 to 61.6%) was observed as vitreousness increased, similar to the decrease in k_d (Table 4.3). Except for Samples 2 and 3 (Table 4.3), all other samples differed significantly ($P \leq 0.05$). The differential association between starch granules and protein matrix in each fraction of endosperm therefore altered the accessibility of starch granules to ruminal bacteria (Rooney and Pflugfelder, 1986; Opatpatanaki *et al*., 1994). With *in situ* measurements, and evaluating the influence of genotype and stage of maturity of maize on ruminal starch degradation, Philippeau and Michalet-Doreau

(1997) reported that ruminal starch degradability was higher for lower vitreous maize compared to higher vitreous maize (61.3 vs. 40.1%). When investigating starch fermentation of maize of different genotypes and processing methods with *in vitro* rumen gas production, De Peters *et al.* (2004) reported that total gas produced was significantly higher at 8 h and 72 h of incubation for floury endosperm compared to vitreous endosperm maize. Opatpatanakit *et al.* (1994) also reported significant differences in gas production of maize of different vitreousness. The PRD results of the current study confirms results of various other authors (Rooney and Pflugfelder, 1986; Firkins *et al*., 2001; McAllister *et al.,* 1993; Opatpatanaki *et al*., 1994; Philippeau and Michalet-Doreau, 1997; Ngonyamo-Majee *et al*., 2008ab; Allen *et al*., 2008; Hoffman and Shaver, 2009) who also reported significantly lower amounts of predicted ruminal degraded starch as vitreousness of maize increased.

The uniform results, irrespective of method of determination (*in vivo*, *in sacco*, *in vitro* disappearance or *in vitro* gas production), provides confidence that as maize vitreousness increase, rate and extent of ruminal disappearance decrease. Results of the current study therefore confirm previously published data.

Linear and quadratic relationships of NIR hardness index values (as obtained by single 2230 nm absorbance) against fractional rate (k_d) of starch degradation and predicted ruminal disappearance PRD were determined. Regression coefficients were obtained for all six maize samples of different vitreousnesses, including popcorn (A) or excluding popcorn (B). Results are shown in Table 4.4.

Table 4.4. Coefficients of determination (r^2) of NIR hardness index values against fractional rate of starch disappearance (k_d) and predicted ruminal disappearance (PRD) of starch in maize of various vitreousness.

		Linear		Quadratic	
		K_d	PRD	K_d	PRD
A	NIR hardness index	0.819	0.946	0.950	0.996
B	NIR hardness index	0.905	0.993	0.911	0.993

A- All maize samples of various vitreousness including popcorn
B- All maize samples of various vitreousness excluding popcorn

When comparing the A NIR hardness index against k_d and PRD, significant inverse linear correlations were observed with determination coefficients (r^2) being r^2 = 0.819

(*P* =0.01, SEM = 0.059) for k_d and 0.946 (*P* = 0.00, SEM = 1.517) for PRD. Quadratic regressions showed even better fits, being 0.950 (*P* = 0.01, SEM = 0.361) for k_d and 0.996 (*P* = 0.00, SEM = 0.515) for PRD (Table 4.4).

Popcorn is not actually used within the animal feed industry and was included in the analyses to obtain disappearance values of extremely vitreous maize. When excluding popcorn from the analysis (B NIR hardness index) linear coefficients of determination were higher than when popcorn was included. Values for r^2 were 0.905 for kd (*P* = 0.01, SEM = 0.037) and 0.993 for PRD (*P* < 0.001, SEM = 0.426). Quadratic responses did not differ much for k_d and PDR.

Because of the potential to predict k_d and PDR values beyond NIR index values of 12.41 (the hardest maize when popcorn is excluded), it was decided to show scatterplots and trendlines of linear and quadratic regressions that included popcorn. Linear regressions are shown in Figures 4.2 and 4.3, while quadratic regressions are shown in Figures 4.4 and 4.5.

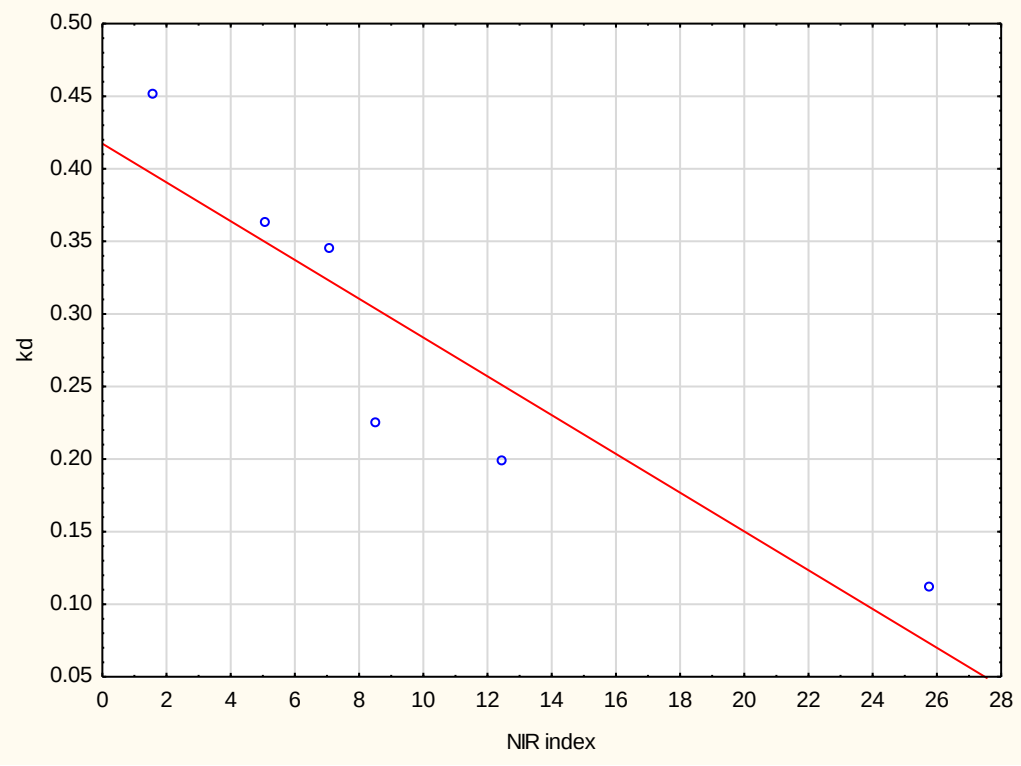

Figure 4.2. Scatterplot and linear regression of fractional degradation rate (k_d) of starch against NIR hardness index (r^2 = 0.819; *P* = 0.013).

In Figure 4.2 it can be seen that the k_d of maize with a NIR hardness index of 1.6 and that of popcorn (NIR hardness index 25.6) was slightly under-predicted, while in those with NIR hardness index of 8.5 and 12.4 it was over-predicted.

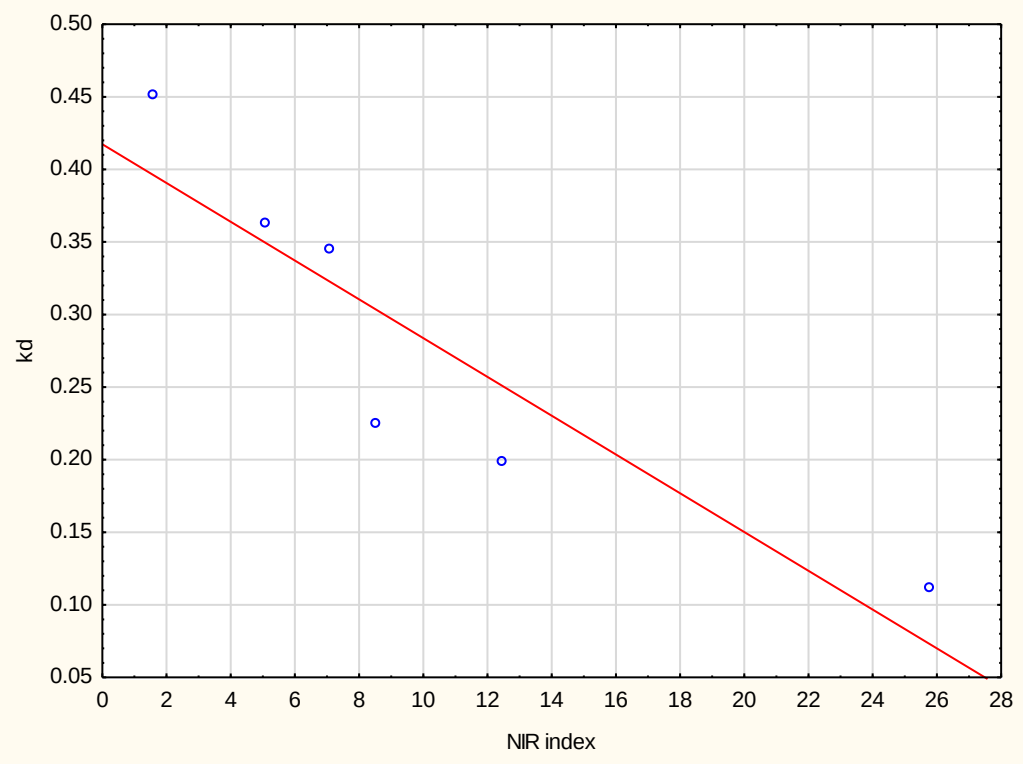

Figure 4.3. Scatterplot and linear regression of predicted rumen disappearance of maize starch (PRD) against NIR index. Disappearance expressed as % of starch at 0 h (r^2 = 0.946; *P* = 0.001).

Figure 4.3 suggests that linear regression predicted PRD fairly accurate from NIR hardness index values, although that of maize with a NIR hardness index of 12.4 was somewhat over-estimated and that of the maize on the extreme sides of the spectrum under-estimated.

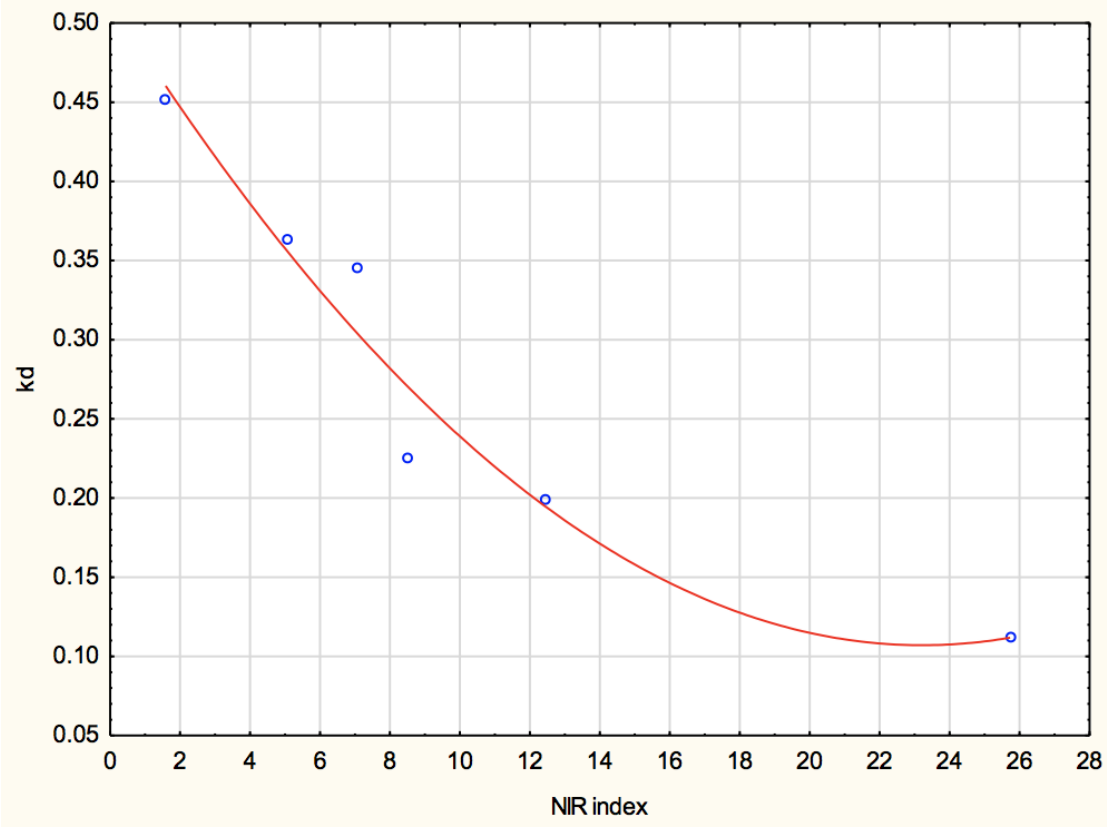

Figure 4.4. Scatterplot and quadratic regression of fractional degradation rate (k_d) of starch against NIR hardness index (r^2 = 0.950; adjusted r^2 = 0.917; *P* = 0.011).

From Figure 4.4 it is apparent that k_d values decreased in a non-linear fashion as NIR hardness index increased. This regression appears to be more accurate than the linear regression, with four of the six observed k_d values being on the trendline. Taking the scale of the Y-axis into consideration, the predicted starch k_d value for maize with a NIR hardness index of 7 was slightly under-predicted, while that for maize with a NIR hardness index of 8.5 was slightly over-predicted.

As for k_d, the PRD values also decreased in a non-linear fashion as NIR hardness index increased. In this case, all the observed values were virtually on the trendline, indicating an excellent fit (Figure 4.5).

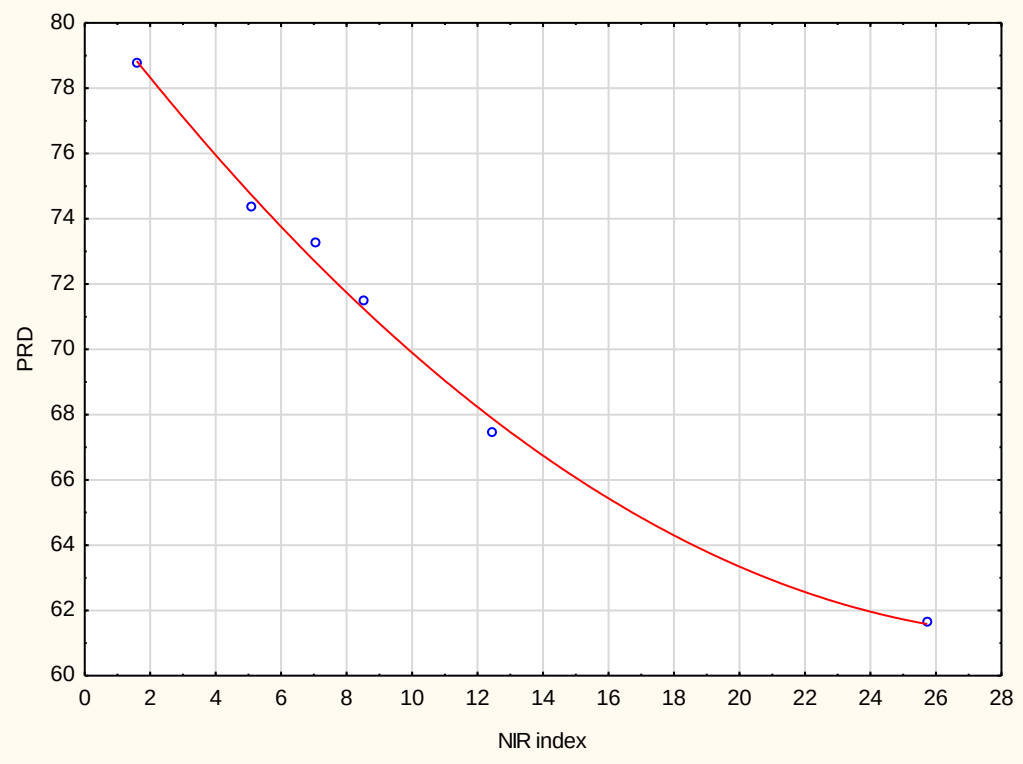

Figure 4.5. Scatterplot and quadratic regression of predicted rumen disappearance of maize starch (PRD) against NIR index. Disappearance expressed as % of starch at 0 h (r^2 = 0.996; adjusted r^2 = 0.993; *P* = 0.001).

The question may arise why a single wavelength was used in the NIR scans and not multiple wavelengths such as being used to predict the chemical composition of feedstuffs. The explanation lies in the fact that, at a wavelength of 2230 nm, reflectance is effectively independent of chemical information in milled samples and varies only with particle size differences (Downey *et al*., 1986; Hoffman *et al*., 2010; Gustin *et al*., 2013; Guelpa, 2015). Furtermore, NIR at a single absorbance of 2230 nm was shown to be accurate to predict hardness of milled of maize (Guelpa, 2015) and wheat (Downey *et al*., 1986). In an earlier chapter of this dissertation (Chapter 3) the accurateness of milled maize hardness determination, as reported by Guelpa (2015), was confirmed. Results of the current study also confirmed a decrease in rate and extent of ruminal degradation as maize vitreousness increased. Therefore, a positive relationship between NIR analyses and ruminal kinetic parameters was to be expected.

4.6 Conclusion

The negative effect of vitreousness of maize on rumen function and degradability of starch has been thoroughly investigated and *in vitro* disappearance results of the current study confirms the increased rate and extent of ruminal starch disappearance as maize vitreousness decreases. However, the rate and extent of maize (especially types with a very low vitreousness) degraded in the rumen might overwhelm the buffering capacity of the rumen and lead to acidosis. The use of high amounts and/or highly fermentable carbohydrates such as highly processed grains are more than often required to sustain animal production, as in the case of high yielding dairy cows. In an effort to prevent metabolic problems with the use of such diets the ruminal kinetic impact of a starch binder and processing needs to be determined.

The global animal feed industry already employs NIR technology extensively as a qualitative and quantitative analytical tool. Almost all modern animal feed mills employ NIR technology, not only to ensure raw material quality, but also to determine rapid, accurate forage analysis. Large investments in accurate calibrations of nutrient components have been made to ensure accurate analysis of both raw materials and forage (e.g. lucerne hay and silages). The mere fact that NIR technology is already available, combined with ease of use, speed, and low cost and infinite application makes this method an ideal one to predict ruminal starch disappearance (PRD) and fractional rate of starch degradation (k_d) for the animal feed industry. Results of this study provide preliminary evidence that significant inverse linear and quadratic relationships exist between NIR hardness index values on the one side and k_d and PRD responses on the other side. The use of NIR technology in an effort to determine rapid, inexpensive k_d and PRD predictions of maize without the use of time consuming, expensive *in vitro* analyses could enable the animal feed industry to formulate more accurately. The more precise ruminal kinetics, as required by the modern mechanistic dynamic models, can therefor be rapidly determined by NIR. As grind size impacts on ruminal starch disappearance kinetics, the impact of processing needs to be established. A correction factor(s) to allow for processing (e.g. grind size) would need to be established to accurately determine ruminal starch k_d and PRD by means of NIR analyses under more practical conditions. Accurate calibrations would also have to be developed.

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CHAPTER 5

The effect of maize vitreousness and a starch binder on *in vitro* rumen kinetics

5.1 Abstract

The objective of the study was to determine the possibility of binding starch in maize with a commercial starch binder to decrease in vitro degradation. One sample each of low (Soft) and high (Hard) vitreous maize, were used and treated with either Bioprotect (BP) or distilled water as control (C) at a rate equivalent to 10 L/tonne. The effect of treatment on starch fermentation was determined in two in vitro trials, viz. a gas production trial and a starch disappearance trial. The experimental design in each trial was a randomized block with runs as blocks. The rate and extent of gas production of the Soft maize was higher ($P < 0.05$) than that of the Hard maize, but BP had no effect on eather vitreousness type. In vitro starch disappearance values were determined after 6, 12 and 24 h of incubation for the same four treatments. Disappearance values were higher ($P < 0.05$) for Soft than Hard maize, but the starch binder had no effect. It was concluded that Bioprotect was not effective to decrease in vitro starch fermentation when maize was milled through a 1 mm screen.

5.2 Introduction

On a global scale, maize (*Zea mays L.*) is the cash crop with the highest production and according to the FOA (2016) the 2014 global harvest amounted to almost 1,1 billion tonnes. Although it may differ from country to country, maize is generally the most common source of starch in ruminant nutrition (Dihman *et al*., 2002; Lopes *et al*., 2009). Maize is grown in most countries where it is used as human food, animal feed and for ethanol production (Ranum *et al*., 2014).

Starch can be defined as: “an alpha-linked-glucose carbohydrate of or derived from plants, animals and microbes from which glucose is released after gelatinization through the use of purified α-amylases and amyloglucosidases that are specifically active only on α-(1-4)- and α-(1-6) linkages” (Hall, 2008, Hall, 2009). The starch content

of feed is determined by enzymatically converting the α-linked-glucose carbohydrate to glucose and then measuring the liberated glucose (Hall, 2008).

Maize starch (and also starch from all other cereal grains) is a major source of energy for ruminant livestock species (Firkins *et al*., 2001; Dihman *et al*., 2002). Dairy cattle consume large amounts of starch (20-40% of diet DM) as a way to increase energy consumption (ME) in support of high milk production (Sniffen, 2004; Patton *et al*., 2011).

In grains, the higher the ratio of vitreous to floury endosperm ratio (V:F), the harder the kernel (Ngonyamo-Majee *et al*., 2008ab). Harder, vitreous endosperm is composed of densely packed starch granules embedded within a complex protein matrix, whereas the softer, floury endosperm contains larger, loosely packed starch granules. The negative effect on ruminant animal performance of high vs. low vitreous maize has been well documented (Firkins *et al*., 2001; Ngonyamo-Majee *et al*., 2008a; Allen *et al*., 2008; Hoffman and Shaver, 2009). Increased kernel vitreousness reduced ruminal *in situ* maize starch degradation (Philippeau and Michalet-Doreau, 1997; Correa *et al*., 2002; Ngonyamo-Majee *et al*., 2008ab) while Taylor and Allen (2005) reported both lower ruminal and total tract starch digestibility (Lopes *et al.,* 2009).

High producing ruminants need the high amounts of starch present in maize endosperm without causing metabolic disorders such as SARA or acute acidosis (Nocek, 1997; Owens *et al*., 1998; Garrett *et al*., 1999). The risks (acidosis) associated with the feeding of high levels of cereal grain have been well documented (Owens *et al.,* 1989). Maize starch is encapsulated in a hard pericarp, which is extremely resistant to microbial degradation in the rumen. Most processing techniques allow RMO greater or easier access to the endosperm therefore increases the rate of fermentation and VFA production (Theurer, 1986; McAllister *et al*., 1990). Increased starch digestion in the rumen, increases propionic acid as a proportion of total VFA in the rumen (Chen *et al*., 1994). Propionic acid is a major gluconeogenic precursor in ruminants, and increasing the proportion of propionic acid might result in:

- A higher net energy absorption from the rumen
- An increase in glucose synthesis by the liver
- A reduction in the use of AA for milk protein synthesis (Theurer, 1986)
- Ultimately improved animal performance.

Increased rate of ruminal starch fermentation would however almost always result in a

decrease in ruminal pH (Rowe *et al*., 1999; Krause and Oetzel, 2006; Penner *et al.,* 2007; Radostits *et al.,* 2007). Ruminal acidosis risks increase when ruminal pH decreases below 6 (Nocek, 1997). The use of highly fermentable starch may also decrease fibre digestion (Leddin *et al.,* 2009), because of a lower ruminal pH. With depressed ruminal pH, NDF digestibility would be severely reduced due to a shift in RMO composition (Leddin *et al.,* 2009). This would result in a shift in VFA production from acetate to propionate (Firkins *et al*., 2001). At a low ruminal pH (< 6), ruminal function is considered to be sub-optimal (Dehghan-banadaky *et al*., 2007). McAllister *et al*. (1991) and Beauchemin *et al*. (1994) also relates the lowering of ruminal pH beneath 6 to rapidly fermentable carbohydrates. Excess fermentation of starch to VFA in the rumen may thus overwhelm the buffering and absorptive capacity of the cow, leading to reductions in ruminal pH. Decreased ruminal pH can further decrease appetite (Britton and Stock, 1987), fibre digestion (Mould *et al*., 1983; Leddin *et al*., 2009) and microbial yield (Strobel and Russell, 1986), leading to decreased energy intake and production. Several studies have also shown that dry matter intake (DMI) decreased significantly when more rapidly available starch sources were fed (McCarthy *et al*., 1989; Moore *et al*., 1992; Aldrich *et al*., 1993).

The method of grain processing further affects the site of digestion of starch in ruminants. Wu *et al*. (1994), found in cows fed steam flaked sorghum that the main site of starch digestion was the rumen. In contrast, when cows were fed dry rolled sorghum, starch was mainly digested in the lower intestine. Table 2.4 (refer to Chapter 2) indicates the major advantages and disadvantages of site of starch digestion as summarized by Rowe *et al.* (1999). According to these authors, it is beneficial to the animal to maximize starch digestion and absorption of glucose from the small intestine. This is based on the energetic efficiency of intestinal digestion being approximately 30% higher than fermentative digestion. Intestinal starch digestion also carries no risk of acidosis as with ruminal fermentative starch digestion (Nocek and Tamminga, 1991). This negative effect of high dietary starch is related to more rapid fermentation and the development of large amounts of lactic acid as primary product and a subsequent lower sub-optimal ruminal pH (Van Soest, 1994). Considerable risk such as laminitis is further associated with fermentative acidosis from high levels of starch reaching the hindgut (McCarthy *et al*., 1989; Godfrey *et al*., 1993; Overton *et al*., 1995; Shabi *et al*., 1999). In contrast, Theurer *et al*. (1999) showed that starch supplementation to the rumen is more beneficial to milk yield compared to post-rumen intestinal supplementation of starch. Huntington (1997) suggests that the primary reason for incomplete starch digestion in the small intestine is due to a lack of adequate

pancreatic amylase, intestinal maltase and isomaltase (Siddons, 1968; Coombe and Smith, 1974) activity and also because of low glucose absorption (Ørskov, 1986; Kreikemeier *et al*., 1991; Tanigushi *et al*., 1995). It has been further shown that the presence of glucose or starch hydrolysate in the small intestine decreases both the secretion of amylase in cattle (Swanson *et al*., 2002) as well as enzyme activity (Kreikemeier *et al*., 1990). Cerrilla and Martínez (2003) suggest that gastrointestinal hormones might thus regulate pancreatic secretion. In general, results indicate that post-ruminal bypass starch utilization is inferior to that of bypass protein (Van Soest, 1994).

Despite variable results, it is clear that it could be beneficial to ruminants to shift some dietary starch digestion from fermentative areas to the small intestine.

Various treatments that had been developed to alter ruminal and total tract starch digestibility as well as site of digestion were shown to be successful and these include cold and hot physical processing methods (Dehghan-banadaky *et al.,* 2007), chemical treatment (Mestres *et al.,* 1991; Robutti *et al.,* 1997; Blandino *et al.,* 2010) and the use of enzymes (Gencoglu, *et al.,* 2010; Weiss *et al.,* 2011; Crosby *et al.,* 2012; McCarthy, *et al.,* 2013). The objectives of these methods are mainly to increase ruminal starch fermentation.

When high amounts of highly fermentable starch are used, the rate and extent of ruminal fermentation could exceed the buffering capacity of the rumen, thereby leading to acidosis. Under such conditions processing techniques would aim to decrease the rate and extent of ruminal starch fermentation in an effort to reduce the risk of metabolic disorders. Based on *in vitro* gas production results (Dunshea *et al*., 2012ab), starch binders were shown to be effective to decrease ruminal starch fermentation of wheat. The active ingredient in these products is a stable non-volatile organic salt that forms complexes with the hydroxyl groups of starch at neutral or slightly acidic conditions (pH 6 to 7), as observed in the rumen (Nocek, 1997; Van Winden *et al*., 2002). These complexes decompose under more acidic (pH 2 to 3) conditions such as in the abomasum and duodenum (Constable *et al.,* 2006), supposedly exposing the starch to be available for enzymatic digestion. However, no current published data exists to confirm if this phenomenon is true of maize starch of variable vitreousness. Despite differences in maize vitreousness, the fermentation rate of wheat is significantly faster than maize (Stock and Britton, 1993; Dunshea *et al.,* 2012ab).

The aim of the current study was to investigate the efficiency of a starch binder on *in*

vitro gas production and *in vitro* starch degradation of high and low vitreous maize.

5.3 Material and methods

In this study *in vitro* rumen starch degradation was determined by two different methods:

- *In vitro* gas production
- *In vitro* starch disappearance

5.3.1 General

Two maize samples, one with a high vitreousness (designated as Hard) and one with a low vitreousness (designated as Soft) were selected from a set of ninety, samples (1kg each) that were collected throughout South Africa, Argentina and Ukraine. These samples originated from larger sets of samples from the 2013 South African harvesting season that had been sent to SAGL (Southern African Grain Laboratory) for regulation analyses, as well as from Ukrainian and Argentinian samples imported to South Africa during 2015. The two samples selected for the current study were selected after ranking them according to vitreousness as determined by NIR hardness index in a previous study (Chapter 3). The NIR hardness index values of the selected samples ranged from 2.33 for the softest to 11.75 for the hardest.

All samples were milled through a standard laboratory mill (Scientec RSA Hammer mill Ser. Nr 372; Centrotec) to pass through a 1 mm screen and were subsequently stored in air tight honey jars while holding samples were vacuum sealed as described in Chapter 4.

5.3.2 Sample preparation

The starch binder used in the current study was Bioprotect (RealisticAgri, Ironbridge, UK). According to Dunshea *et al*. (2012ab) the effect of Bioprotect is maximized at 8 L/tonne of grain to effectively bind wheat starch in the rumen. Due to unavailability of previous peer reviewed documented research with starch binder treatment of maize, the optimum dosing rate reported by Dunshea *et al.* (2012ab) for the treatment of

wheat was used. According to the manufacturers (Jefferis, 2016) and from results of Dunshea *et al.* (2012ab) no negative effects were observed with grains when application rate was higher than 8L/tonne. A slightly higher than reported minimum dosage rate reported for wheat by Dunshea *et al*. (2012ab) was thus used to ensure even distribution and effectiveness of the starch binder. The specific gravity of Bioprotect was determined as 1.257 (25 mL = 31.43 g). The delivery mass obtained from a micro spray bottle that was used in the trial to treat the maize was determined to be 0.1 g/spray. The volume of each spray thus resulted in a delivery of 0.08 mL. Duplicate amounts of 16 ± 0.01 g per sample from one hard and one soft maize sample were accurately weighed. Subsequently one of each hard and soft sample was treated with two sprays of Bioprotect, resulting in 0.16 mL per treated sample (BP). This was equivalent to a treatment dose of 10 L/tonne. Accurate digital moisture determination of all samples were done in duplicate at 120°C by a Radwag moisture analyzer (NDC Technologies, Irwindale, California, USA. Model Max50/NH).

5.3.3 Rumen fluid collection

Incubations with rumen fluid from ruminally cannulated cows on different diets showed similar ranking orders of different starches with respect to extent and rate of degradation (Huhtanen and Sveinbjörnsson, 2006). Therefore, the specific diet of the donour cow(s) is not that important when rumen fluid is used for *in vitro* incubations, as long as the diet is the same for all donour cows and consistent for the duration of an *in vitro* trial (Weimer, 2017).

Fresh rumen fluid was collected prior to each *in vitro* run from three ruminally cannulated lactating Holstein dairy cows at 07h00 in the morning before the morning feeding for the *in vitro* gas production trial. The same protocol was used to collect rumen fluid for the *in vitro* starch disappearance trial, with the exception that two cows were used in the latter trial. All rumen collections were done in accordance to the rumen extraction protocol of the University of Stellenbosch and were approved by the Stellenbosch University's Animal Ethics Committee (reference: SU-ACUD16-00157). Cows were from the Welgevallen Experimental Farm's herd of the University of Stellenbosch, South Africa. The cows received the same TMR and were fed at the same time of the day as described in Chapter 4.

Rumen fluid was collected transported and processed in the lab similarly as previously

described (Chapter 4). However, in this study the collected rumen fluid of different animals were not pooled.

At collection, the pH of the rumen fluid was also recorded (Table 5.1). Observed variation in rumen fluid pH between different animals and runs could be attributed to variation in feed intake due to selection, dominance and reproductive cycle on the day prior to collection (Weimer, 2017).

Table 5.1. Rumen pH of collected rumen fluid.

	Cow	Run 1	Run 2	Run 3
In vitro gas production	1	5.09	5.93	6.73
	2	5.94	6.03	6.35
	3	6.44	5.85	6.52
In vitro starch disappearance	1	6.73	6.87	5.39
	2	6.35	6.80	5.49

5.3.4 *In vitro* solutions

All *in vitro* substrate samples were incubated in a buffered incubation medium containing a rumen fluid inoculum, as described by Goering and Van Soest (1970) and Van Soest and Robertson (1991) in a previous Chapter (Chapter 4). All procedures, amounts of substrate, volume of rumen liquor, reagents and equipment used, were similar as described for the starch disappearance trial in Chapter 4 (Goering and Van Soest, 1970; Van Soest and Robertson, 1991).

5.3.5 In vitro gas production

The digestion of feedstuffs by ruminal microorganisms (RMO) produces gas (McBee, 1953), and gas measurements have long been used to measure the extent and kinetics of *in vitro* degradation (Menke *et al.,* 1979; Beuvink *et al.,* 1992; Pell and Schofield, 1993).

The gas production method used in this study was based on the Reading Pressure

Technique (RPT) (Mauricio *et al*., 1999). This method is used for the evaluation of feed *in vitro* and it is based on a semi-automated gas production technique as described by Mauricio *et al.* (1999).

Glass vials, with a nominal volume of 120 mL, were used for incubation of the maize samples. The exact volume of each vial was required for accurate determination of headspace volume and was thus predetermined. Blank vials, containing buffered rumen inocula, without substrate, were also prepared to obtain reagent blanks that would be used later to correct for gas produced by the rumen fluid alone (Mauricio *et al*., 1999).

Amounts of 300 ± 10 µg of each prepared maize sample (binder moisture adjusted) plus a 20 mm magnetic stirrer bar with a volume of 0.2 mL were placed into each vial. A surgical syringe was used to add 40 mL of the buffered medium into each vial. Vials were gassed with a gentle stream of CO_2 and 20 mm rubber stoppers were placed on the vials without pushing them in all the way. The vials were then placed in a prewarmed (39.6^{o}C) incubation chamber. After the incubation buffer had been reduced, the vials were reopened and 10 mL of the strained rumen fluid was added to each vial while again gassing with CO_2. Thereafter the rubber stoppers were pushed into the vials and sealed with 20 mm aluminum crimp caps. The vials were subsequently transferred to magnetic stirrer plates inside the incubation chamber and the contents were stirred at a low speed. A timer (set at 15 minutes per hour) was used to automatically control stirring time. Surgical needles (40 mm, 21 gauge), fitted to pressure transducers, were inserted through the rubber stoppers. The transducers were linked to the pressure logging system and the pressure (psi) inside each vial was recorded every 10 seconds for the entire incubation period of 48 hours, thereby creating 17280 data points for each sample. The logging system was custom built by Eagle Technology (Pty) Ltd. (Cape Town, South Africa). To prevent the possibility of limited gas transfer from the fluid to the headspace, excess pressure buildup (psi $\geq$ 9) was avoided by releasing pressure on regular intervals from the vials. The incubation chamber temperature was maintained at a constant of 39.6^{o}C throughout the entire incubation period.

5.3.5.1 Conversion of gas pressure to gas volume

The pressure transducers records pressure (psi). The following linear regression

equation that was developed for the specific setup in the Department of Animal Sciences, University of Stellenbosch, was used to convert gas pressure to gas volume:

$$Y = \frac{[(1000 ((0.0977 X) C)]}{OM}$$

Where: Y = Gas volume (mL/g OM)
X = Gas pressure (psi)
C = Vial head space (mL)
OM = Organic matter (mg)

5.3.5.2 Estimation of kinetic coefficients

The Solver option in Microsoft Office Excel and a non-linear model that included a lag phase was used to calculate the kinetic coefficients from the gas volume data. The model used was based on a modified version of that described by Ørskov and McDonald (1979).

$$Y = b (1 - e^{-c(t-L)})$$

Where: Y = gas volume at time t
b = total gas production (mL g^{-1} DM)
c = rate of gas production (h^{-1})
t = incubation time (hours)
L = lag time (hours)

5.3.6 *In vitro* starch disappearance

According to gas production results by Hoffman *et al.* (2012), where they evaluated the rate of ruminal starch degradation of different grains, degradation commences and increases rapidly after four hours of incubation and peaks at approximately six hours. Thereafter it declines to almost insignificant levels at 24 hours of incubation. For the current *in vitro* starch disappearance trial, incubation times were thus set at 6, 12 and 24 hours respectively. Results from three runs were recorded, thereby creating 72 vectors for each vitreousness class (2) x treatment (2) x incubation time (3) x animal

blocks (6). Sample allocation for each of the three runs is presented in Appendix 3.

Amounts of 300 ± 10 µg of each prepared maize sample (binder moisture adjusted) plus a 20 mm magnetic stirrer bar were placed into 250 mL Nalgene plastic bottles according to the schedule in Appendix 3. Buffered rumen liquor inoculated medium was used for the *in vitro* incubations. Reagent medium preparation was according to Goering and Van Soest (1970) and Van Soest and Robertson (1985) and was the same as described in Chapter 4. The procedures and volume of rumen liquor and reagents were also the same as in the gas production study described earlier. After the reagents had been added, gassed with CO_2 and the incubation buffer had been reduced, rubber stoppers were placed on the containers. The containers were subsequently transferred to the incubation chamber and placed on a magnetic stirrer plate. A timer (set at 15 minutes per hour) was used to control stirring time automatically.

The temperature of the incubator chamber was maintained at 39.6°C throughout the entire incubation periods of 6, 12 and 24 hours. The incubated samples were removed from the incubation chamber at the appropriate times as per schedule (Appendix 3) and immediately placed on ice for 30 minutes to stop fermentation. Cooled samples were subsequently stored at 4 °C for starch analyses.

5.3.6.1 Starch analysis

In the current study, starch analyses for both the substrate and the *in vitro* digesta residues were based on the method as described in by Hall (2009) in Chapter 3.

5.4 Statistical analysis

A non-linear model was used to derive potential gas production values (b), gas production rates (c) and lag times (L). These parameters were then subjected to main effects ANOVA with the aid of Statistica, version 13 (Stat Soft, Inc., Tulsa, USA). Main effects were treatments (Hard vs. Soft maize and C vs. BP) and blocks (rumen fluid from different cows). Fisher's least significant difference (LSD) method was used in ANOVA to create confidence intervals for all pairwise differences between factor level means. Significantly different means were separated with a Bonferroni test. Significance was declared at $P \leq 0.05$ and tendencies at $P \leq 0.10$.

5.5 Results and discussion

According to Taysom (2013) trying to mimic *in vivo* digestibility with *in vitro* techniques is neither the goal, nor a realistic expectation. *In vitro* analysis can only evaluate the potential digestibility of a feedstuff (Taysom, 2013).

5.5.1 *In vitro* gas production

When incubation of a feedstuff with rumen fluid takes place *in vitro*, fermentation of carbohydrates will produce gasses (CH_4 and CO_2), short chain fatty acids and microbial cells. Gas is produced as a result of carbohydrates that are fermented to volatile fatty acids propionate, acetate and butyrate (Getachew *et al.*, 1998). Gas is produced in larger quantities when carbohydrates are fermented to acetate and butyrate. When carbohydrates are fermented to propionate, a relatively smaller amount of gas will be produced (Hungate, 1966; Van Soest, 1994; Getachew *et al.*, 1998). The production and ratios of volatile fatty acids are largely dependent on the diet of a ruminant animal (Bergen & Yokoyama, 1977; Van der Merwe & Smith, 1991). Various studies have shown a high correlation between dry matter disappearance and *in vitro* gas production and starch availability of grains (Menke *et al.*, 1979; Xiong *et al.*, 1990; Blummel and Ørskov, 1993; Opatpatanakit *et al.*, 1994).

The effects of the starch binder treatment and maize vitreousness on *in vitro* gas production parameters are shown in Figure 5.1. From Figure 5.1 it appears that the total volume of gas and the rate of gas produced are higher and faster for soft (low vitreous) maize compared to hard (high vitreous) maize. The higher amount and faster rate of gas production of lower vitreous maize supports results from various other authors who found similar results in terms of either gas production or ruminal starch disappearance (Firkins *et al*., 2001; Philippeau and Michalet-Doreau, 1997; Correa *et al*., 2002; Taylor and Allen, 2005; Ngonyamo-Majee *et al*., 2008ab; Allen *et al*., 2008; Hoffman and Shaver, 2009). Total amounts and rate of gas produced in this study are in line with results of De Peters *et al.* (2003) who also reported similar *in vitro* gas production curves from processed and unprocessed maize.

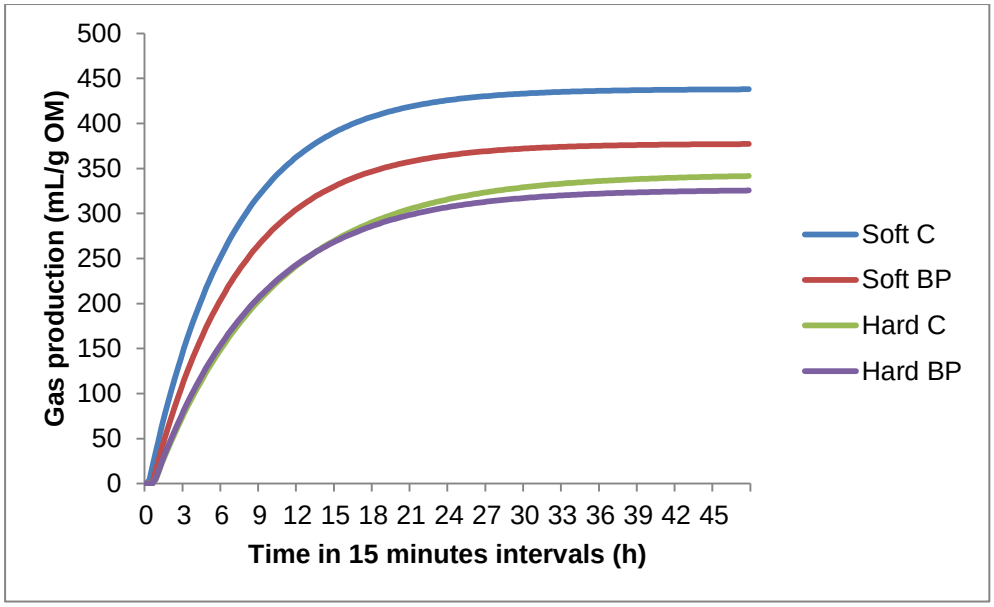

Figure 5.1 The effect of Bioprotect treatment of maize on *in vitro* gas production of maize with different vitreousness indices.

Analyzing only the main effects (Soft vs. Hard), the mean values of gas production parameters for maize with different vitreousness indices are presented in Table 5.2. Despite a relatively high degree of variation for volume of gas produced, Table 5.2 shows the effect of vitreousness on gas volume ($P = 0.036$) and rate of gas production ($P = 0.001$). *In vitro* gas production has been shown to be a reliable estimator of ruminal fermentation kinetics, for forages (Cone, 1994), as well as for grains (Chai *et al.*, 2004). The latter authors also showed that both rate and amount of gas production are positively correlated with ruminal starch degradation (Cone 1998a; Pashaei *et al.,* 2010). Furthermore, Ngonyamo-Majee *et al.* (2008a) showed a strong negative correlation between vitreousness and starch degradation in the rumen. Harder, vitreous endosperm is composed of densely packed starch granules embedded within a complex protein matrix, whereas the softer, floury endosperm contains larger, loosely packed starch granules. This strong starch-protein matrix of high vitreous starch, limit RMO access to kernel starch and is responsible for slower ruminal starch fermentation rates compared to lower vitreous starch (Rooney and Pflugfelder, 1986; McAllister *et al*., 1993; Opatpatanaki *et al*., 1994). In accordance, with *in vitro* gas production, Dunshea *et al.* (2012ab) also reported increasing ruminal fermentation rates from sorghum to maize compared to hard wheat with the fastest rate recorded for soft wheat. Gas production rates for wheat differed significantly from that of maize ($P <$

0.001), while a tendency ($P < 0.10$) between hard and soft wheat was reported. Results from this study confirm that the total volume of gas produced by *in vitro* fermentation is lower with high vitreousness compared to low vitreous maize.

Table 5.2. The effect of vitreousness on *in vitro* gas production parameters of maize with different vitreousness indices.

Item	Treatment[1]		SEM	*P*
	Soft	Hard		
b[2]	407.8[a]	335.1[b]	22.64	0.036
c[3]	0.037[a]	0.029[b]	0.000	0.001
L[4]	2.39	3.49	0.440	0.092

[a,b]Means within rows with different superscripts differ ($P < 0.05$).
[1]Treatments: Soft = low vitreous maize; Hard = high vitreous maize.
[2]b = Gas volume produced (mL/g OM).
[3]c = Rate of gas produced (mL/h).
[4]L = Lag time (h).

Apart from vitreousness, processing also affects starch degradation in the rumen. With *in vitro* gas production techniques, De Peters *et al.* (2003) demonstrated significant increased ruminal rate and extent of starch fermentation with processing of maize. Processing techniques allow RMO greater or easier access to the endosperm, thus increasing the rate of fermentation and VFA production (Theurer, 1986; McAllister *et al.,* 1990; Eastridge, 2006).

Increasing starch fermentation in the rumen increases propionic acid as a proportion of total VFA in the rumen (Chen *et al*., 1994). Effective processing to offset the slower rate and extent of fermentation associated with high vitreousness (Table 5.2) is therefore more important when high vitreous maize is used.

The effect of Bioprotect treatment on *in vitro* gas production parameters of maize (analyzing only the main effects Bioprotect (BP) vs. Control (C)) is presented in Table 5.3.

Table 5.3. The effect of Bioprotect treatment on *in vitro* gas production parameters of maize.

Item	Treatment[1]		SEM	*P*
	C	BP		
b[2]	390.9	351.9	24.97	0.286
c[3]	0.032	0.033	0.002	0.766
L[4]	2.65	3.23	0.470	0.390

[a,b]Means within rows with different superscripts differ ($P < 0.05$).
[1]Treatments: C = maize treated with distilled water (equivalent to 10 L/tonne); BP = maize treated with Bioprotect (equivalent to 10 L/tonne).
[2]b = Gas volume produced (mL/g OM).
[3]c = Rate of gas produced (mL/h).
[4]L = Lag time (h).

No significant effects were observed between treatments in this study. These results are in contrast with results of Dunshea *et al.* (2012ab) who showed that the treatment of wheat with a starch binder decreased both total gas volume produced ($P < 0.05$) and the rate of gas production ($P < 0.001$). The rate of gas produced by *in vitro* fermentation of maize compared to wheat was significantly slower, thereby confirming the ruminal fermentation differences between different starches (Dunshea *et al.,* 2012ab). Although not significantly different, in agreement with Dunshea *et al.* (2012ab) a slightly lower (numerical) total amount of gas was produced with BP compared to C.

Results from Table 5.3 may appear to be in contrast with results presented in Figure 5.1. However, a relatively high degree of variation between means of the results probably had and effect on the results. The variation observed was probably due to the limited number of repetitions (n = 6).

The effect of Bioprotect treatment on gas production parameters of low vitreous maize (analyzing the main effects Soft C vs. Soft BP) is presented in Table 5.4.

Slight numerical decreases regarding the extent of gas produced and the rate of gas production with the starch binder treatment were observed for soft maize in this study. In contrast, Dunshea *et al.* (2012ab) found significant effects with wheat. Again, the limited number of replications probably caused a lack of significance and more research in this area may be warranted. In the current study, it can thus not be concluded from the gas production results alone that the starch binder was effective

to slow *in vitro* fermentation of soft maize significantly.

Table 5.4. The effect of Bioprotect on *in vitro* gas production parameters of low vitreous maize.

Item	Treatment[1]		SEM	*P*
	Soft C	Soft BP		
b[2]	438.2	377.3	33.51	0.255
c[3]	0.038	0.036	0.002	0.597
L[4]	1.81	2.96	0.429	0.115

[a,b]Means within rows with different superscripts differ ($P < 0.05$).
[1]Treatments: Soft C = low vitreous maize treated with distilled water (equivalent to 10 L/tonne); Soft BP = low vitreous maize treated with Bioprotect (equivalent to 10 L/tonne).
[2]b = Gas volume produced (mL/g OM).
[3]c = Rate of gas produced (mL/h).
[4]L = Lag time (h).

The effect of the Bioprotect treatment of high vitreous maize alone (analyzing main effects Hard C vs. Hard BP) on *in vitro* gas production parameters is presented in Table 5.5.

Table 5.5. The effect of Bioprotect on *in vitro* gas production parameters of high vitreous maize.

Item	Treatment[1]		SEM	*P*
	Hard C	Hard BP		
b[2]	343.5	326.6	7.09	0.153
c[3]	0.027	0.03	0.002	0.287
L[4]	3.48	3.49	0.028	0.895

[a,b]Means within rows with different superscripts differ ($P < 0.05$).
[1]Treatments: Hard C = high vitreous maize treated with distilled water (equivalent to 10 L/tonne); Hard BP = high vitreous maize treated with Bioprotect (equivalent to 10 L/tonne).
[2]b = Gas volume produced (mL/g OM).
[3]c = Rate of gas produced (mL/h).
[4]L = Lag time (h).

No significant differences were found between treatments. The mean amount of gas

produced with Hard BP was numerically lower compared to Hard C. Less variation was observed when comparing treatment effects of Hard maize alone compared to that of Soft maize. It is merely hypothesized that the higher levels of variation observed in the Soft maize results (as indicated by the SEM values) might be an indication that more replications could have resulted in significant results for Soft maize rather than for Hard maize. Even when taking variation into account, Figure 5.1 may suggest less of an effect in Hard maize than in Soft maize. This may have been expected, as the rate and extent of hard maize starch degradation in the rumen is already inherently slow (Philippeau and Michalet-Doreau, 1997; Correa *et al*., 2002; Ngonyamo-Majee *et al*., 2008ab; Dunshea *et al.,* 2012ab). The ability of the starch binder to bind slow fermenting starch to further decrease ruminal starch degradation therefore appears to be limited.

The effect of Bioprotect on *in vitro* gas production parameters and interactions of maize with different vitreousness indices is presented in Table 5.6.

Table 5.6. The effect of Bioprotect on *in vitro* gas production parameters of maize with different vitreousness indices.

Item	Treatment[1]				SEM	*P*
	Soft C	Soft BP	Hard C	Hard BP		
b2	438.2	377.3	343.5	326.6	32.07	0.112
c3	0.038[a]	0.036[a]	0.027[b]	0.030[b]	0.002	0.008
L4	1.81	2.96	3.48	3.49	0.623	0.227

[a,b]Means within rows with different superscripts differ ($P < 0.05$).
[1]Treatments: Soft C = low vitreous maize treated with distilled water (equivalent to 10 L/tonne); Soft BP = low vitreous maize treated with Bioprotect (equivalent to 10 L/tonne); Hard C = high vitreous maize treated with distilled water (equivalent to 10 L/tonne); Hard BP = high vitreous maize treated with Bioprotect (equivalent to 10 L/tonne).
[2]b = Gas volume produced (mL/g OM).
[3]c = Rate of gas produced (mL/h).
[4]L = Lag time (h).

When taking all the effects of treatment and vitreousness in account, no interactions were observed (Table 5.6). Only the rate of gas production for Soft C and Soft BP differed significantly from Hard C and Hard BP ($P < 0.05$). Mean values for extent of gas and rate of gas produced were, however, 16.1% and 5.5% lower for Soft BP compared to Soft C respectively (Table 5.6). This difference is also visible in Figure 5.1. Although mean values for Soft BP showed decreased gas production parameters

(numerically) compared to Soft C, results were non-significant possibly due to high variation (Table 5.6). When looking at results of Dunshea *et al.* (2012ab), who reported a significant effect of starch binder treatment of wheat (a rapidly fermentable grain) on starch degradation rates, a similar effect was expected, but not observed, for Soft BP maize in the current study.

The almost similar rates and volume of gas produced with Hard C vs. Hard BP indicate the ineffectiveness of a starch binder to effectively bind slow fermentable starch within the rumen environment. The differences observed between results of this study compared to that of Dunshea (2012ab) could be attributed to the strong resistant protein matrix and relative large amylose content of maize compared to small grain, which would affect fermentability by limiting microbial access to starch granules (McAllister *et al.,* 1993; Huntington, 1997). Streeter *et al.* (1993) reported that, although grinding of cereal grains exposed the interior of endosperm cells to enzymatic degradation, the starch granules remained embedded in a protein matrix. With maize, this protein matrix was resistant to rumen microbial fermentation, whereas in barley the matrix was susceptible to fermentation (Streeter *et al.,* 1993). The starch binder treatment of slow fermentative starch such as from high vitreous maize in this study also did not succeed to lower the rate and extent of ruminal fermentation as measured by *in vitro* gas production parameters.

5.5.2 *In vitro* starch disappearance

By investigating interactions between treatment, incubation time and vitreousness, no treatment*incubation time (P = 0.950), treatment*vitreousness (P = 0.086), treatment*vitreousness (P = 0.804) or treatment*vitreousness*incubation time (P = 0.982) significant interactions were observed; therefore the main effects of Treatment and Vitreousness could be interpreted separately (Table 5.7).

From Table 5.7 it is evident that the BP treatment did not result in lower starch disappearance over the total 24 h period. This is in contrast with Dunshea *et al.* (2012ab) who found significant effects with wheat. Differences were, however, observed between maize types in terms of vitreousness, where starch disappearance was higher ($P < 0.05$) in Soft than in Hard.

Table 5.7. The effect of treatment and vitreousness on mean starch disappearance over 24 hours.

Item			SEM	*P*
	Treatment			
Starch disappearance for Treatment (%)	BP	C		
	73.3	70.8	3.51	0.621
	Vitreousness			
Starch disappearance for Vitreousness (%)	Soft	Hard		
	77.2	66.9	3.51	0.049

When evaluating only the fixed effect time of incubation, all time points (6, 12, 24) differed from each other. This was to be expected as according to Hoffman *et al.* (2012) maize starch degradation commences and increases rapidly after four hours of incubation and peaks at approximately six hours. Thereafter it declines to almost insignificant levels at 24 hours of incubation.

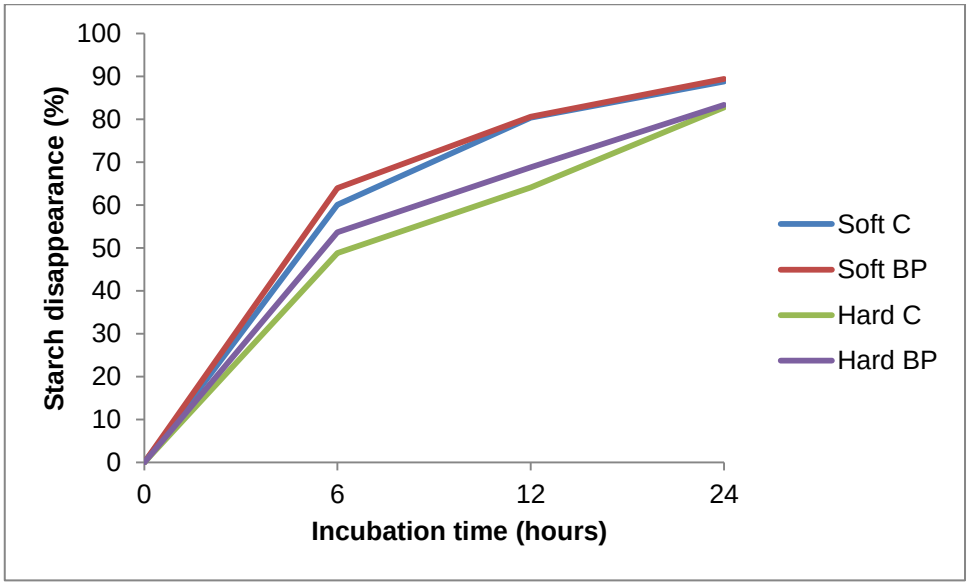

Figure 5.2 The effect of Bioprotect treatment of maize on *in vitro* starch disappearance of maize with different vitreousness indices.

Results for *in vitro* starch disappearance after 6, 12 and 24 h of incubation for Soft C, Hard C, Soft BP and Hard BP maize are presented in Figure 5.2. Disappearance values are presented in Table 5.8.

In both Figure 5.1 (previous section) and Figure 5.2 it can be seen that fermentation and starch degradation increased rapidly until about 6 h of incubation after which rates apparently started to decline. From about 24 h, values started to approach an asymptote regarding gas production, but regarding starch disappearance, and particularly in the case of the Hard maize, digestibility values were still increasing.

Table 5.8. The effect of Bioprotect on *in vitro* starch disappearance of maize with different vitreousness indices.

Time	Treatment[1]				SEM	*P*
	Soft C	Soft BP	Hard C	Hard BP		
6 h	60.1[a]	64.0[a]	48.8[b]	53.7[b]	2.872	0.009
12 h	80.4[a]	80.6[a]	64.1[b]	68.8[b]	2.571	0.006
24 h	88.8[a]	89.4[a]	82.8[b]	83.4[b]	1.708	0.024

[a,b]Means within rows with different superscripts differ (P < 0.05).
[1]Treatments: Soft C = low vitreous maize without Bioprotect; Soft BP = low vitreous maize treated with Bioprotect (equivalent to 10 L/tonne); Hard C = high vitreous maize without Bioprotect; Hard BP = high vitreous maize treated with Bioprotect (equivalent to 10 L/tonne).

At least 80% of the starch was completely degraded after 24 h incubation (Table 5.8). Starch disappearance differences between means of Soft BP and Hard BP were 10.3, 11.8 and 6 percentage units (P < 0.05) for the 6, 12 and 24 h incubation times respectively. For the same time intervals, starch was 11.3, 16.3 and 8 percentage units lower for Hard C compared to Soft C, respectively. It thus appears that *in vitro* starch degradation rates were, irrespective of treatment or incubation time, significantly (P < 0.05) slower for Hard compared to Soft maize (Figure 5.2). After 24 h of incubation, significantly less (*P* < 0.05) starch was degraded with Hard maize compared to Soft maize (Table 5.8). As indicated in the previous section (Figure 5.1), fermentation of Hard maize continued at a lower rate compared to Soft maize. Based on the high correlation between gas production and starch degradation (Cone, 1995; Chai *et al.*, 2004), it is postulated that the *in vitro* starch disappearance of the Hard maize used in the current study would have plateaued at a lower level than that of the Soft maize,

had the trial continued to 48 h. Lopes *et al.* (2009) in agreement, reported a linear decrease in ruminal starch degradation of 24-33 and 18-20 percentage units between soft and hard maize at 8, 16 h incubation times respectively.

Regarding the effect of starch binder, *in vitro* degradability values of Soft C were not different to those of Soft BP, regardless of incubation time (Table 5.8). The same observation is valid for the Hard C and Hard BP treatments.

The results of this study indicated an ineffectiveness of the starch binder to bind maize starch at 1 mm maize grind size, irrespective of vitreousness. Despite only three time points, the results further indicated that the starch binder could not alter maize starch degradation rate. More time points would need to be investigated. Dunshea *et al.* (2012ab), in contrast, reported significantly slower rates of starch degradation when wheat was treated with the same starch binder. No other published data regarding the effectiveness of the starch binder to effectively bind maize starch could be found. It is therefore hypothesized that the reason for the conflicting results between Dunshea *et al.* (2012ab) and the current study is likely related to the type of grain and their inherent differences in rate of fermentation. The fermentation rate of maize, irrespective of vitreousness, is significantly slower than that of wheat (Dunshea *et al.,* 2012ab). Streeter *et al.* (1993) further reported that after processing of grain, the protein matrix of maize was still resistant to rumen microbial fermentation, whereas in barley the matrix was susceptible to fermentation. The current study supports this, as it appears that the starch binder had little effect on altering *in vitro* rumen kinetics of maize of different vitreousness.

5.6 Conclusion

In agreement with various other published results, gas production rate and extent of ruminal starch fermentation was slower and lower with high compared to low vitreous maize. These results were confirmed by the *in vitro* starch disappearance trial as well as results of Chapter 4 from this dissertation. However, to obtain sufficient data that can be used in the animal feed industry, a larger number of maize samples accros the spectrum of hardness index should be investigated.

The treatment of maize with a commercial starch binder did not significantly change *in vitro* rate or amount of gas produced in this study. *In vitro* results regarding disappearance of starch from maize with different vitreousness, incubated at various

times, also indicated the inability of the starch binder to affect the extent of starch degradation. In this study, the starch binder treatment of maize did not bind starch of maize milled through a 1 mm screen effectively.

The current study provides preliminary results regarding the ability of a commercial starch binder to effectively bind maize starch in the rumen. Despite insignificance in this study, numerical tendencies were observed with the use of a starch binder. Further research to fully understand the starch binding capacity of low vitreous maize is required. The combined effect of starch binder treatment and grinding processing on ruminal disappearance of low vitreous maize starch at different time intervals needs to be determined.

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CHAPTER 6

The effect of maize particle size and a starch binder on *in vitro* rumen kinetics

6.1 Abstract

The objective of the study was to determine the effects of a commercial starch binder treatment and particle size on in vitro fermentation characteristics of known low vitreousness maize. Maize was hammer milled to pass either through 1 mm (1) or 4 mm (4) screens. Treatment was with either distilled water (C) or a starch binder (Bioprotect, RealisticAgri, Ironbridge, UK) (BP). The processed and binder treated samples therefor yielded four treatments: 1 mm processed and distilled water treated (C1), 1 mm processed and Bioprotect treated (BP1), 4 mm processed and distilled water treated (C4) and 4 mm processed and Bioprotect treated (BP4). Rumen fermentation parameters were calculated after in vitro starch disappearance for all 4 treatments after 0, 3, 6, 12, 24 and 48 h of incubation were determined. Main effect particle size reduction significantly (P = 0.05) increased the fractional rate of ruminal disappearance (k_d) from 0.06 /h to 0.20 /h, while main effect BP treatment showed a tendency (P = 0.06) towards reduced k_d. Predicted ruminal disappearance (PRD) showed similar results and interactions with main effect particle size (P = 0.00) and main effect PRD (P = 0.00) between 1 vs. 4 and C vs. BP respectively. Both k_d and PRD did not differ between C1 and BP1, but in this study C4 showed a significantly (P =0.02) higher k_d and significantly (P =0.00) higher PRD compared to BP4. Results of this study indicate that the reduction in particle size of maize with hammer mill processing changes rumen starch fermentation characteristics. Results further indicate that the treatment of 4 mm milled maize with a commercial starch binder can change rumen fermentation kinetics.

6.2 Introduction

Starch from maize is an important source of dietary energy for lactating dairy cows and other ruminants (Blasel *et al.,* 2006). However, the availability of starch to enzymatic hydrolysis influences how cereal grains are digested by ruminants (Huntington 1997;

Crocker *et al.,* 1998; Offner *et al.,* 2003). Starch granules are the form of storage of carbohydrate by most cereal plants. The susceptibility of the starch granules to enzymatic hydrolysis influences the digestion of starch and thus determines the amount of energy obtained by the animal. The physical and chemical characteristics of cereal starches are complex (Gallant *et al.,* 1992). Maize possesses endosperm with both a floury and vitreous region (DePeters *et al.,* 2007). A strong continuous protein matrix embeds starch granules of vitreous maize endosperm (Opatpatanakit *et al.,* 1994). Starch granules of floury endosperm are more susceptible to grain processing and accessible to digestive enzymes (Kotarski *et al.,* 1992; Huntington 1997). The stability of the protein matrix varies with cereal type. In maize, the protein matrix resists microbial fermentation whereas in barley the matrix is susceptible to fermentation (McAllister *et al.,* 1993). Thus, the protein matrix surrounding the starch endosperm contributes to the digestibility of dietary starch. With gas production, Opatpatanakit *et al.* (1994), showed significant differences among cereal grain species and were ranked in terms of gas production potential as: wheat > triticale > oats > barley > maize > rice and sorghum. In a meta-analysis evaluating 290 data sets, Moharrery *et al.* (2014) summarized fractional rates of ruminal starch degradation (k_d) and found it to be low for maize (0.08 %/h), high for barley (0.40 %/h) and very high for oats and wheat (ranging from 0.60 t0 1.04 %/h). Opatpatanakit *et al.* (1994) also reported significant differences in gas production between maize of different vitreousness. This was confirmed with results from Chapter 4 of this dissertation.

It is well documented that processing of maize, such as grinding, cracking, rolling, roasting, popping, exploding, flaking etc. for ruminant animals that are fed high levels of concentrates enhances *in vitro* starch utilization (Theurer, 1986). This improvement appears to be mainly due to an increase in the degradability of starch in the rumen, resulting in an enhanced total tract digestibility and thus improved energy availability from the maize (Owens *et al.,* 1997; Kim *et al.,* 1996). Physical processing decreases the particle size of maize, thus increasing the surface area available for microbial attack (Bowman and Firkins, 1993; Offner *et al.,* 2003), and enhancing the rate and extent of ruminal degradation of starch and VFA production (Theurer, 1986; McAllister *et al*., 1990; Kim *et al.,* 1996, Callison *et al*., 2001, Ramos *et al.,* 2009).

In contrast, where very high fermentable starch, such as wheat (Van Soest, 1994), or where very high amounts (Leddin *et al*., 2009) of highly processed grain are fed, the objective of processing would be to decrease the rate and extent of ruminal fermentation in an effort to decrease the risk of fermentative acidosis. An increased

rate of ruminal starch fermentation will almost always result in a decrease in ruminal pH (Rowe *et al*., 1999). The risk of ruminal acidosis increases when ruminal pH decreases below 6 (Nocek, 1997). Starch binders were shown with *in vitro* gas production studies by Dunshea *et al.* (2012ab) as an effective processing technique to decrease ruminal starch fermentation of wheat. The active ingredient in these binders (Bioprotect) is a stable non-volatile organic salt that forms complexes with the hydroxyl groups of starch at neutral or slightly acidic conditions (pH 6 to 7), as observed in the rumen (Nocek, 1997; Van Winden *et al*., 2002). According to the suppliers, these complexes supposedly decompose under more acidic (pH 2 to 3) conditions, such as in the abomasum and duodenum (Constable *et al.,* 2006), thus exposing the starch to be available for enzymatic digestion lower in the digestive tract. The specific mode of action of Bioprotect was discussed in an earlier chapter of this dissertation (Chapter 5). Despite numerical differences with soft maize reported earlier in this dissertation (Chapter 5), no significant binding of maize starch was found in either *in vitro* gas production or *in vitro* starch disappearance values of 1 mm milled maize. However, the interaction of grind size and starch binder was not investigated. As the standard grind size of maize in the South African feed industry is currently 4 mm, the aim of the study was to determine the effect of a starch binder (Bioprotect) and grind size (1 mm vs. 4 mm) on ruminal fermentation kinetics of low vitreous maize.

6.3 Material and methods

6.3.1 General

The batch of maize used in this study was produced under moderate climatic conditions and irrigation and, according to its NIR hardness index, was found to fall in the low vitreous category, as explained in a previous chapter (Chapter 3) of this dissertation. The maize was harvested during the June 2016 harvest season in South Africa. Vitreousness of the maize was predetermined by NIR analysis at a single 2230 nm absorbance and V:F determination with the use of a single 106 µm sieve, which was shown earlier in this dissertation (Chapter 3) and by other authors (Burden, 2010; Guelpa, 2015; Cruywagen, 2016) to be reliable predictors of vitreousness. It was also determined earlier in this dissertation (Chapter 3) that a V:F ratio of < 1 (Guelpa, 2015), and/or NIR hardness index < 7 of milled maize, indicate low vitreousness (Cruywagen, 2016). Triplicate samples of the maize used in the current study were analysed for

vitreousness and the mean values were 2.96 ± 0.007 (SD) for NIR hardness index and 0.786 ± 0.024 (SD) for V:F. It is thus clear that the maize was of low vitreousness.

6.3.2 Maize treatment

Although the current feed industry standard in South Africa is to mill maize through a 4 mm mesh, Hall (2009) suggested a 1 mm grind size to establish a standard procedure for starch analysis of animal feeds. Mertens (2005) further warned that it is important that particle size of the sample does not inhibit digestion if the research objective is to measure the intrinsic rate of digestion of chemical components and also recomended that samples should be ground to pass through a 1 mm screen. For the current study, it was decided to use two grind sizes to determine the effect of both particle size and Bioprotect on *in vitro* starch disappearance. Two samples were therefore hammer milled (Drotsky M20, RSA) to pass through a 4 mm screen and a secondary two samples through a standard laboratory mill (Scientec RSA Hammer mill Ser. Nr 372; Centrotec) to pass through a 1 mm screen. Subsequently, samples of each grind size were equally and similarly treated with Bioprotect (RealisticAgri, Ironbridge, UK) and distilled water (dH_2O) as described in Chapter 4 to render four treatments: 1 mm dH_2O treated (C1), 4 mm dH_2O treated (C4), 1 mm Bioprotect treated (BP1) and 4 mm Bioprotect treated (BP4). The DM content of all the treated samples was determined (drying at 105°C for 24 h) to enable accurate calculation of the samples weighed out into the incubation vessels. The processed and treated samples were stored in airtight honey jars for analysis and incubation.

6.3.3 Rumen fluid collection

In this study, fresh rumen fluid from two cannulated lactating Holstein dairy cows was collected, transported, handled and pooled (Chapter 4) as described in Chapters 4 and 5 of this dissertation. The pH of the rumen fluid varied between 6.1 and 6.4 between animals and runs. All rumen collections were done in accordance to the rumen extraction protocol of the University of Stellenbosch and was approved by the Stellenbosch University's Animal Ethics Committee (reference: SU-ACUD16-00157).

6.3.4 *In vitro* solutions

All *in vitro* samples were incubated in a buffered incubation medium containing a rumen fluid inoculum, as described by Goering and Van Soest (1970) and Van Soest and Robertson (1991) and explained in Chapter 4.

6.3.5 *In vitro* starch disappearance

For the current *in vitro* starch disappearance trial, the same incubation times were used as in the *in vitro* disappearance trial described in Chapter 4 were used. Statistical concepts indicate that regression coefficients are determined more accurately when the same number of observations are collected once at more times rather than multiple observations collected at fewer times (Mertens, 2005). In a meta-analysis, Maccarana *et al.* (2016) concluded from 30 published articles that the majority of *in vitro* fermentation studies did not exceed 48 h, therefore observations were determined at 0, 3, 6, 12, 24 and 48 h per run. The temperature of the incubator chamber was maintained at constant temperature of 39.6^{o}C for the duration of the trial. With each run, reagent blank samples, without substrate, were incubated and analyzed to correct for starch in the rumen fluid (Mertens, 2005). All incubated samples were removed from the incubation chamber at the appropriate times (Appendix 4) and immediately placed on ice for 30 minutes to stop fermentation. Cooled samples were subsequently stored at 4^{o}C for starch analyses. Mertens (2005) further suggested that at least three observations are needed for each parameter to be estimated in the digestion model. Results from four runs were recorded in the current study. Sample allocation for each of the four runs is presented in Appendix 4.

To ensure that more representative substrate samples are taken (due to coarser than 1 mm grind size of some samples), amounts of 600 ± 10 µg of each prepared maize sample (binder moisture adjusted) were weighed out into 250 mL Nalgene bottles. The incubation medium was accordingly adjusted and 80 mL of the buffered medium and 20 mL of rumen liquor inoculant were added to each bottle. All other procedures, prepared buffered medium (Goering and Van Soest, 1970; Van Soest and Robertson, 1991) and equipment used were similar as described for the starch disappearance trials in Chapters 4 and 5.

6.3.6 Starch analysis

In the current study, starch analyses for both the substrate and the *in vitro* digesta residue were based on the method as described by Hall (2009) and in Chapter 3.

6.3.7 Estimation of kinetic coefficients

Estimations for ruminal kinetic coefficients were fitted and calculated by first order non-linear modeling as described in Chapter 4. Predicted effective ruminal starch disappearance (PRD) was calculated according to Batajoo and Shaver (1998) and Bal and Shaver (2006) as in Chapter 4.

6.4 Statistical Analysis

A non-linear model (Ørskov and McDonald, 1979) was fitted to the disappearance data to determine starch disappearance at time t, dissolvable starch (a), potential degradable starch (b), fractional rate of degradation (c) and lag time (L). The first deritives were used in a secondary model (Batajoo and Shaver, 1998; Bal and Shaver, 2006) to determine predicted ruminal starch disappearance (PRD). All the kinetic coefficients were then subjected to two-way analysis of variance (ANOVA). Data were analyzed using REML to ensure consistency of *P*-values on VEPAC of STATISTICA version 13 (Stat Soft, Inc., Tulsa, USA). A factorial ANOVA approach was used to determine possible interactions between processing and treatment of kinetic parameter means. Fisher's least significant difference (LSD) method was used in ANOVA to create confidence intervals for all pairwise differences between factor level means. Significance was declared at $P \leq 0.05$ and tendencies at $P \leq 0.10$.

6.5 Results and discussion

According to Taysom (2013), trying to mimic *in vivo* digestibility with *in vitro* techniques is neither the goal, nor a realistic expectation. *In vitro* analysis can only evaluate the potential and relative degradability of a feedstuff (Taysom, 2013; Powel-Smith *et al.,* 2015).

By evaluating the rate and extent of ruminal starch degradation of different grains with gas production, Hoffman *et al.* (2012) reported that degradation commences and increases rapidly after four hours of incubation and peaks at approximately six hours. Thereafter, it declines to almost insignificant levels at 24 hours of incubation. In a study evaluating the effect of processing techniques on *in vitro* degradability of maize, Lee *et al.* (2002) used 2, 6, 12, 24, 48 hours incubation to determine rate of degradation. According to Mertens (2005), Hall (2017) and Weimer (2017) six time points of incubation is sufficient to determine fractional rates of disappearance.

The maize used in this study fell within the low vitreousness category and therefore the amount of prolamine should have been relatively low for maize (Fox and Manley, 2009). The expectation was thus that the maize used in this study would be relatively soluble in rumen fluid (Rowe *et al*., 1999). Because the maize starch granules were not completely surrounded by zein, ruminal degradation could not be as limited as with high vitreous maize (Kotarski *et al*., 1992; Johnson *et al*., 1999; Gibbon *et al*., 2003).

Results of the *in vitro* non-linear parameters, predicted starch disappearance and PRD are presented in Table 6.1. The a-fraction (readily soluble and rapidly degradable) was lower ($P < 0.05$) for the 1 mm than for the 4 mm milled maize (Table 6.1). The a-fraction is typically assumed to have infinite rate of degradation (Ørskov and McDonald, 1979) or an extremely fast rate such as 2.0 - 4.0 /h (Sniffen *et al.,* 1992). In contrast, the slowly degradable fraction (b) was higher ($P < 0.05$) for finely ground maize compared to the coarse ground maize. These results are difficult to explain, but is likely related to differences in amylose and amylopectin processing characteristics due to the different screen sizes. Amylopectin is soluble in water at room temperature, while amylose is not (Green *et al.,* 1977). It is hypothesized that with course grinding (4 mm), the floury endosperm that contains more amylopectin separates readily from the vitreous endosperm and therefore contributes to a higher soluble fraction expressed as percent of total starch compared to the 1 mm size. No differences were, however, observed between C and BP regarding the a- and b-fractions (Table 6.1). Reported values for the a-fraction have also been highly variable both between and within feeds (Nocek and Tamminga, 1991; Offner *et al.,* 2003). Obeserved lag times did not show any particular pattern, but were lower than 0.45 h for all samples, indicating a short period of time before fermentation commences (Table 6.1).

Table 6.1. The effect of maize grind size and a starch binder on *in vitro* non-linear parameters and predicted ruminal disappearance of starch

Item	Treatment[1]				SEM	*P*
	C1	BP1	C4	BP4		
a[2], %	20.5[a]	18.7[a]	28.5[b]	30.6[b]	0.621	<0.001
b	75.6[a]	75.6[a]	59.5[b]	54.2[b]	1.315	<0.001
k_d	0.198[a]	0.194[a]	0.081[b]	0.038[c]	0.010	<0.001
lag, h	0.41[a]	0.44[b]	0.39[a]	0.40[a]	0.010	<0.02
PRD[3]	76.0[a]	73.87[a]	60.3[b]	49.7[c]	1.121	<0.001

[a-d]Means in the same row with different superscripts differ ($P \leq 0.05$).
[1]Treatments were maize ground through a 1 mm or 4 mm sieve and treated with either Bioprotect (BP) or distilled water (C).
[2]Non-linear parameters a = starch that disappeared after soaking in distilled water for 30 minutes; b = potentially degradable starch; k_d = fractional rate of degradation; lag = time before fermentation commences.
[3]PRD = Predicted ruminal disappearance.

The effect of maize grind size and starch binder (Bioprotect) on *in vitro* starch starch disappearance over time is presented in Figure 6.1.

Despite the initial lower a-fraction (Table 6.1) observed with the 1 mm grind size, rapid degradation within the first 2 to 3 h of incubation is evident compared to the 4 mm grind size. Although significantly ($P < 0.05$) slower than in the case of 1 mm, the 4 mm milled maize also showed an increase in degradation from 2 to 20 h of incubation and thereafter plateauing off to an asymptote (Figure 6.1). The C4 and BP4 treated maize tended to plateau at more or less 40-50 h of incubation while for both C1 and BP1 an asymptote was reached after approximately 24 h of incubation. In this study, both C4 and BP4 therefore degraded slower ($P < 0.05$) than C1 and BP1 (Figure 6.1 and Table 6.1). Various other authors (Theurer, 1986; McAllister *et al*., 1990; Kim *et al.,* 1996; Callison *et al*., 2001; Lee *et al.*, 2002, Hoffman *et al.*, 2012) investigating the effect of maize processing on ruminal kinetic parameters, reported similar degradation curves and increased rate of fermentation, as maize processing increased. With *in situ* data, Lykos and Varga (1995) showed a linear inverse relationship between particle size obtained after processing and ruminal starch fermentation of maize. Cone *et al.* (1989) also reported that starch disappeared faster when particle size was reduced from 1 to 0.1 mm.

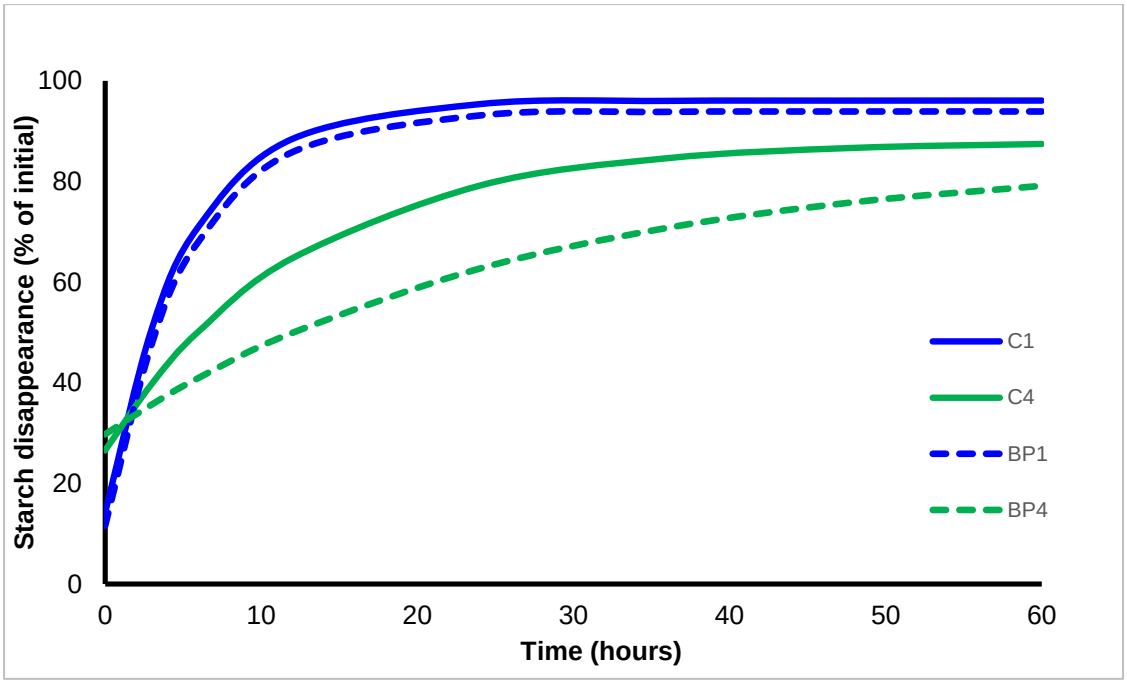

Figure 6.1 The effect of maize grind size and a starch binder (Bioprotect) on *in vitro* starch starch disappearance.

Predicted ruminal starch disappearance (PRD) followed the same trend as k_d and was lower ($P < 0.05$) with a 1 mm grind size compared to 4 mm. The absolute PRD values observed in this study are similar to those of Ramos *et al.* (2009) who reported a range of 41.9 to 65.8 % for maize of various vitreousness milled through a 3 mm screen size. Philippeau *et al.* (1999), evaluating starch degradability of 14 maize genotypes ground through a 3 mm sieve, reported a range of 41 to 78 %. Meta-analysis data of 158 maize ruminal starch degradation studies revealed a mean PRD of 57.4 % (Moharrery *et al.,* 2013). The effect of grinding on PRD in this study was similar as previously reported for ground (3 mm or 4 mm sieve) maize of similar vitreousness (Philippeau *et al.,* 1999; Correa *et al.,* 2002). The differences in absolute PRD values of the current study compared to other studies could be explained by differences in grind size, vitreousness and passage rates used. In a study evaluating particle size of dry milled maize (4.8, 2.6 and 1.2 mm), Callison *et al*. (2001) reported that fine grinding of maize greatly increased ruminal starch degradability. Decreasing the particle size of maize affected true ruminal digestibility of NSC quadratically (49.8, 46.5, and 87.0%, respectively) (Callison *et al*., 2001). With a *in sacco* study, evaluating maize vitreousness and particle size, Ramos *et al.* (2009) also reported a significant ($P < 0.001$) reduction in both ruminal and intestinal digestion with increased particle size. The uniform results

in literature of faster fermentation rate and extent of starch degradation with higher degrees of processing confirms the hypothesis that physical processing decreases the particle size of maize, thus increasing the surface area available for microbial attack (Bowman and Firkins, 1993; Offner *et al.,* 2003), and thereby enhancing the rate and extent of ruminal degradation. Results of the current study support this theory.

McAllister *et al*. (1993) proposes that the protein and carbohydrate matrix (containing amylose and amylopectin) within a cereal kernel is more important in determining the extent of ruminal starch degradation than the physical or chemical properties of starch. Other *in vitro* studies also indicated that the protein matrix is the major factor responsible for differences in ruminal degradation of maize and barley (Huhtanen & Sveinbjörnsson, 2006). Although the V:F endosperm ratio of low vitreous maize is lower than high vitreous maize, the amount and type of prolamine in maize (Hoffman and Shaver, 2009) is higher and different to wheat (DePeters *et al.,* 2007). This suggests that, even when vitreousness of maize is low, a protein matrix is still present and will therefore impact on degradability results of maize compared to wheat and barley (McAllister *et al.,* 1993; Opatpatanakit *et al.,* 1994; DePeters *et al.,* 2007).

Although no differences in k_d and PRD were evident between the C1 and BP1 treatments, both k_d and PRD values were lower ($P \leq 0.05$) for BP4 compared to C4 (Figure 6.1 and Table 6.1). The fact that Bioprotect had no effect on k_d or PRD in finely ground maize (1 mm), but significant effects in the coarser maize (4 mm) are difficult to explain. The expectation was that a higher degree of processing would expose more fine granules of amylopectin and amylose for the Bioprotect to attach to. The ability of the binder to form more or stronger hydrogen bonds with the amylopectin and amylose of 4 mm grind size compared to 1 mm is not fully known and cannot be readily explained. It is hypothesized that the processing of 4 mm maize did not break the starch granules as effectively as in the case of 1 mm processing and that this phenomenon could be related to the results. It is also hypothesized that with course grinding (4 mm), the floury endosperm that contains more amylopectin separates readily from the vitreous endosperm and enable Bioprotect to bind effectively to the free amylopectin. This, as a percent of total starch, might thus result in decreased starch degradation. In this study Bioprotect thus showed a similar binding capacity in 4 mm processed maize as was observed with Bioprotect treated wheat by Dunshea *et al.* (2012ab).

The observed differences in k_d and PRD between treatments of the current study could also be related to the differences in processing equipment. Richards *et al.* (1995) found

that different feed samples had almost the same *in vitro* rate of starch disappearance ranking order when a Wiley mill was used for grinding the samples, but this was not the case with an Udy mill. Therefore the Wiley mill was recommended, and using the Wiley mill resulted in similar starch degradation irrespective of screen size (1 mm vs. 2 mm). In the current study, a laboratory hammer mill (Scientec RSA Hammer Mill Ser. Nr 372; Centrotec) was used for the 1 mm grinding, which could have had an impact on the results. The hammer mill used to process the 4 mm maize might not have disrupted the amylose adequately and therefore the binder essentially only bound to amylopectin as discussed. Differences between the effective binding of wheat that was observed by Dunshea *et al.* (2012ab) and the 1 mm ground maize of the current study could be attributed to differences in amylose content (McAllister *et al.,* 1993; DePeters *et al.,* 2007) and processing equipment used for maize and wheat (Richards *et al.,* 1995). In the mildly acidic conditions of the *in vitro* incubation medium, Bioprotect (containing three double bonded oxygen atoms) could have formed stronger multi-links with the higher amounts of amylopectin of the starch chains of 4 mm processed maize compared to 1 mm and BP4 showed similar responses to binder treated wheat as observed by Dunshea *et al.* (2012ab).

6.6 Conclusion

Despite slight differences in absolute values within the literature, as well as observed in this study, the ruminal rate and extent of degradation was similar to most reported results, namely an increase when maize particle size is reduced. By reducing maize particle size, the surface area available for microbial attack is increased, and therefore enhances the rate and extent of ruminal degradation. Results of this study indicate that both the rate and extent of ruminal disappearance of low vitreous maize is significantly higher when particle size is reduced by milling through a 1 mm screen compared to 4 mm.

A decreased *in vitro* fractional rate of disappearance (k_d) and predicted ruminal degradability (PRD) observed for the 4 mm maize indicate that Bioprotect treatment effectively decreased the rate and extent of ruminal disappearance of coarse low vitreous maize. When high amounts of low vitreous maize is fed, results of the current study suggest that the associated risk of metabolic acidosis could be decreased by treatment with a starch binder (10 L/tonne grain) and processing through a 4 mm screen.

Although a positive effect of the starch binder with coarse maize (4 mm) was shown *in vitro*, the effect on maize starch digestion in the lower digestive tract is unknown. If the starch binder protects some of the starch against ruminal degradation and releases it again under the acidic conditions in the small intesine, a higher efficiency of starch utilization would be expected, manifested in increased starch digestibility and animal performance. There is thus a need to determine the effect of Bioprotect treatment of coarse maize (4 mm), as used in the feed industry, on total tract starch digestibility and production parameters of lactating dairy cows.

6.7 References

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CHAPTER 7

The effect of the treatment of low vitreous maize with a starch binder on total tract digestion and production parameters of lactating dairy cows

7.1 Abstract

An experiment was conducted to evaluate the effect of a commercial starch binder (Bioprotect RealisticAgri, Ironbridge, UK) treatment of low vitreous maize (Zea mays L) on total tract starch digestion and production response in lactating dairy cows. Six primiparous Holstein dairy cows (165 ± 45 DIM at trial initiation) were randomly assigned to a replicated 2 × 3 change over design with 14 d periods; the first 11 d of each period were for diet adaptation, followed by 3 d for sampling and data collection. Treatment diets contained untreated (C) or starch binder treated (BP) maize (10 L/tonne of maize). The TMR diets consisted of maize (385g/kg of DM), lucerne hay (296g/kg of DM), a commercial high protein-mineral-vitamin concentrate (203g/kg of DM), molasses meal (14g/kg of DM) and straw (101g/kg of DM). Maize was hammer milled to pass a 4 mm screen prior to treatment and mixing. Total tract starch digestibility was determined where iNDF was used as an internal marker to estimate faecal excretion. Dry matter intake, milk yield, 4% fat corrected milk yield and energy corrected milk yield were unaffected by treatment. Milk solids content, ratios and yield, as well as MUN and somatic cell count did not differ between treatments. Although apparent total tract dry matter and crude protein digestibility did not differ between treatments, apparent total tract starch digestibility decreased significantly when maize was treated with the starch binder compared to untreated maize. It was concluded that the starch binder used in the current study may not be effective for the treatment of maize when the aim is to increase the efficiency of starch utilization.

7.2 Introduction

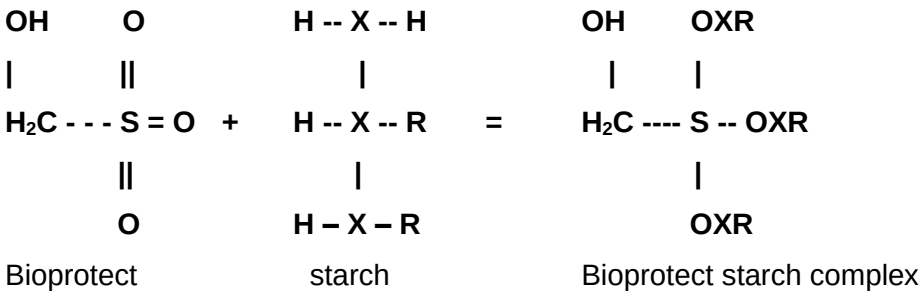
Starch is a major source of energy for ruminant livestock species (Firkins *et al*., 2001; Dihman *et al*., 2002). Dairy cattle consume large amounts of starch (20-40% of diet DM) in order to increase energy consumption (ME) in support of high milk production (Patton *et al*., 2011). Maize (*Zea mays L.*) is a primary source of starch and is the largest cash crop produced internationally and by far the most widely used energy source in ruminant feeds (Dihman *et al*., 2002; Lopes *et al*., 2009).

With all grain, the higher the ratio of vitreous to floury endosperm, the harder the kernel (Ngonyamo-Majee *et al*., 2008ab). Harder, vitreous endosperm is composed of densely packed starch granules embedded within a complex protein matrix, whereas the softer, floury endosperm contains larger, loosely packed starch granules (Abdelrahram and Hoseney, 1984). The negative effect on ruminant animal production of high vs. low vitreous maize, have been well documented (Firkins *et al*., 2001; Ngonyamo-Majee *et al*., 2008a; Allen *et al*., 2008; Hoffman and Shaver, 2009). Increased kernel vitreousness reduced ruminal *in situ* maize starch degradation (Philippeau and Michalet-Doreau, 1997; Correa *et al*., 2002; Ngonyamo-Majee *et al*., 2008ab) while Taylor and Allen (2005) reported both lower ruminal and total tract starch digestibility. Earlier in this dissertation (Chapter 5) the reduction of fractional rate (k_d) and extent (PRD) of ruminal starch disappearance was also confirmed. Increased vitreousness of maize is also associated with a reduction in small intestinal pancreatic starch digestion (Ngonyamo-Majee *et al*., 2008ab).

Under normal conditions, the bovine rumen maintains a pH of 6.5 to 7.2 (Nocek, 1997; Van Winden *et al.,* 2002). In contrast, the small intestine (abomasum and duodenum) of the ruminant is more acidic with a pH of 2 to 3 (Constable *et al.,* 2006). Some commercial products (Bioprotect, RealisticAgri, Ironbridge, UK) have been shown to protect highly fermentable starch, such as wheat, against ruminal degradation (Dunshea *et al.,* 2012ab). The active ingredient in these products is a stable non-volatile organic salt that complexes with the hydroxyl groups of starch at neutral or slightly acidic conditions (pH 6 to 7), as observed in the rumen (Nocek, 1997; Van Winden *et al*., 2002). These complexes supposedly decompose under more acidic (pH 2 to 3) conditions, such as in the abomasum and duodenum, thereby exposing the starch to be available for enzymatic digestion. Lower intestinal starch degradation is mainly driven by pancreatic amylase secretion (Cerrilla and Martínez, 2003).

A typical starch can be represented as:

According to the suppliers, Bioprotect is in the form H_2CO-S (ONa)(OH) and has great affinity with the hydrogen bonds of starches. In mildly acidic conditions of the rumen (when highly fermentable carbohydrates are fed), complex Bioprotect starch structures are formed containing R-X-O-CH_2-SO_3 Na linkages. Alternatively, Bioprotect, containing three double bonded oxygen atoms, can form multi-links with a starch chain:



In a trial with sheep measuring the effect of Bioprotect on total tract starch digestibility, Gonzalez *et al.* (2014) demonstrated that reducing rumen fermentation of wheat does not decrease total tract starch digestibility. These authors hypothesized that adequate enzymatic (pancreatic amylase) starch digestion in the small intestine of sheep sustain or increase total starch digestibility of Bioprotect treated wheat compared to maize or untreated wheat (Gonzalez *et al.,* 2014).

Increased amounts of starch could also escape ruminal degradation by increased rumen fluid dilution rate (Cerrilla and Martínez, 2003). The dilution rate of rumen fluid is higher with long than ground roughage (Hodgeson and Thomas, 1975) and is related to the higher amount of time spent ruminating. In a study with lambs fed different lengths of roughage, the amount of ground maize starch that passed to the duodenum of sheep doubled when ground straw was replaced with long straw (Thompson and Lamming, 1972; Thompson, 1973). Ørskov *et al*. (1969) earlier reported similar results.

It has also been suggested that the digestion of starch post rumenly is used more efficiently than that digested in the rumen (Nocek and Tamminga, 1991). Ruminant animals may be capable of digesting large amounts of starch in the small intestine through an adaptation in the activity of the host carbohydrases (Janes *et al*., 1985).

In contrast, the capacity of the ruminant small intestine to digest large amounts of starch is questionable (Waldo, 1973; Croome *et al*., 1992), due to:

- Relative low levels of pancreatic amylase, intestinal maltase and isomaltase (Siddons, 1968; Coombe and Siddons, 1973; Coombe and Smith, 1974).
- Relative low glucose absorption capacity (Ørskov, 1986; Kreikemeier *et al*., 1991; Tanigushi *et al*., 1995).

Both decreased amylase secretion (Swanson *et al*., 2002) and enzyme activity (Kreikemeier *et al*., 1990) have been found in the presence of glucose or starch hydrolysate in the bovine small intestine. Cerrilla and Martínez (2003) suggest that gastrointestinal hormones might thus regulate pancreatic secretion.

Kreikemeier *et al*. (1990) reported a higher amylolytic activity when a high protein lucerne hay diet was fed vs. a grain diet, with equal amounts of energy. This could be related to the stimulation of the pancreas by the protease-sensitive cholecystokinin releasing peptide due to the presence of protein in the intestine (Fushiki *et al*. 1989). It is thus possible that pancreatic enzyme secretion in ruminants might be mediated by a monitor peptide (Fushiki *et al*. 1989). Results from Kreikemeier *et al*. (1990) similarly suggest that the amount of protein in the diet could play an important role in starch digestion in the small intestine.

The method of grain processing also affects the site of digestion of starch in ruminants. Wu *et al.* (1994), reported that the main site of starch digestion with cows fed steam flaked sorghum was the rumen. In contrast, when cows were fed dry rolled sorghum, more starch was shifted to the small intestine for digestion. According to Rowe *et al.* (1999) it is beneficial to the animal to maximize the digestion of starch and absorption of glucose from the small intestine. This is based on the energetic efficiency of intestinal digestion being approximately 30% higher than fermentative digestion (Nocek and Tamminga, 1991). Intestinal starch digestion also carries no risk of acidosis as with ruminal fermentative starch digestion. Theoretically, fermentation in the rumen is less energetically efficient (80%) than enzymatic digestion in the small intestine (97%) (Harmon and McLeod, 2001; Huntington *et al.,* 2006). However,

digestion in the large intestine is even less energetically efficient (44%) than digestion in either the rumen or small intestine. Digestion and absorption of starch in the small intestine occurs in three phases (Huntington *et al.,* 2006):

1) Pancreatic α-amylase initiates starch breakdown in the duodenum,
2) Absorption occurs at the brush border membrane through the action of the brush border carbohydrases, and
3) Glucose is transported out of the intestinal lumen and into portal circulation.

Propionate is almost completely removed from portal blood by the liver (Kittivachra *et al.,* 2007). Within the liver, propionate serves as a major substrate for gluconeogenesis, and accounts for 45-60% of the glucose formed in ruminants. Gluconeogenesis from non-sugars accounts for 40-55% of glucose; takes place outside mammary gland and involves breakdown of protein (Tucker, 2000). The availability of glucogenous compounds in high producing cows is an important feed nutrition factor. The principal precursor of milk lactose is blood glucose (Huntington *et al.,* 2006). In the cow 60-70% of the blood glucose taken up is utilized for lactose synthesis in the alveolar epithelial cell in the mammary gland (Tucker, 2000). Certain microorganisms in the cow's rumen produce volatile fatty acids (VFA) such as acetate, propionate and butyrate as end products of fermentation of cellulose and other sugars. Milk is further isotonic with blood. A reduction in glucose availability is a limiting factor in milk production. Decreased glucose leads to decreased lactose synthesis, water secretion into milk and milk volume. Therefore, there is a high correlation between milk yield and glucose uptake from blood (Kittivachra *et al.,* 2007).

Bovine amylase appears to be pH sensitive as ruminal starch digestion has been shown to improve by the addition of buffers (Wheeler *et al*., 1977). This negative effect of high dietary starch is related to more rapid fermentation and the development of large amounts of lactic acid as primary product and a subsequent lower sub-optimal ruminal pH (Van Soest, 1994). Considerable risk such as laminitis is further associated with fermentative acidosis from high levels of starch reaching the hindgut (McCarthy *et al*., 1989; Godfrey *et al*., 1993; Overton *et al*., 1995; Shabi *et al*., 1999). In contrast, Theurer *et al*. (1999) showed that starch supplementation to the rumen is more beneficial to milk yield than post-ruminal intestinal supplementation of starch.

Despite variable results, it appears that it could be beneficial to ruminants to shift some starch digestion from fermentative areas to the small intestine. The apparent ruminal

(Dunshea *et al*., 2012ab) and total tract (Gonzalez *et al.,* 2014) benefit with the treatment of wheat with a starch binder clearly indicates the potential of reducing metabolic problems associated with the use of high volumes of highly fermentable starch diets.

According to Huhtanen & Sveinbjörnsson (2006), all *in vitro* and *in sacco* methods currently used to estimate *in vivo* ruminal starch degradability and the fractional rate of starch degradation are problematic. According to these authors, only total tract starch digestibility using animals can be measured with relative small errors. By investigating data of 32 commercial herds, Powel-Smith *et al.* (2015) in support, also concluded that *in vitro* starch disappearance at 7 h was not related to total tract starch digestibility ($r^2 = 0.00$), and that TTSD was not correlated with surface area of particles or mean particle size of the dry ground maize that had been fed. To obtain reliable and accurate estimates of energy availability for ruminants, Powel-Smith *et al.* (2015) therefore recommends reliance only on total tract digestion measurements. Similarly, Schuling *et al.* (2016) evaluated both *in vitro* and *in situ* rumen starch degradation methods and also found that *in vitro* starch degradation was not related ($r^2 = 0.19$, $P < 0.002$) to *in vivo* results.

In a previous trial (described in Chapter 6), it was found that a commercial starch binder (Bioprotect) dit not affect *in vitro* starch disappearance of finely ground maize (1 mm) but decreased disappearance ($P < 0.05$) in coarser maize (4 mm). Since maize that is ground through a 4 mm sieve is typically used in the South African feed industry, it was hypothesised that Bioprotect would decrease starch digestion in the rumen, thus making more starch available for digestion in the small intestine and thereby increase total tract starch digestion in dairy cows. A study was thus conducted to test this hypothesis and the subsequent effect on milk production response in lactating Holstein cows.

7.3 Material and methods

7.3.1 General

The effect of the treatment of low vitreous maize with a starch binder on TTSD, production and metabolic responses of lactating dairy cows were investigated due to

the global importance of maize as a major glucogenic source to high producing ruminants. Low vitreous maize was used due to the faster rate of ruminal fermentation compared to high vitreous maize (Chapter 5). Maize used in this study was produced under moderate climatic conditions and irrigation, and was found to fall in the low vitreous category as shown in a previous chapter (Chapters 3 and 6). The maize was harvested during the June 2016 South African harvest season.

The maize used in this study was the same as had been used in Chapter 6. Vitreousness of the maize was therefore similarly predetermined by NIR analysis at a single absorbance of 2230 nm and V:F determination with the use of a single 106 µm sieve, which was shown (Chapter 3) to be reliable methods to predict vitreousness of milled maize (Burden, 2010; Cruywagen, 2016). Hardness results of the specific maize used in this study was discussed in Chapter 6. It was determined previously in this dissertation (Chapter 3) that a V:F ratio of < 1 (Guelpa, 2015), and/or NIR hardness index < 7 of milled maize, indicate low vitreousness (Cruywagen, 2016).

7.3.2 Maize treatment

The current feed industry standard in South Africa is to mill maize through a 4 mm mesh. All the maize used in this study was subsequently hammer milled (Drotsky M20, RSA) through a 4 mm screen prior to treatment and/or mixing.

A linear response with the treatment of wheat with a starch binder (Bioprotect, RealisticAgri, Ironbridge, UK), revealed that an application of 8 L/tonne of grain yielded optimal results (Dunshea *et al.* (2012a). The manufacturer of Bioprotect indicated no negative or positive (apart from cost) effect of higher dosage rates than 8 L/tonne (Jefferis, 2016). Maize used in the treatment diet of this study was therefore treated with a handheld fertilizer sprayer at a single dosing rate of 10 mL Bioprotect/kg maize. The higher than minimum recommended treatment dosage was used to ensure even distribution and effective mixing. Maize treatment was applied daily prior to TMR mixing, while the maize of the control diet was treated with water at a similar dosage of 10 L/tonne.

7.3.3 Trial design and data collection

Six primiparous Holstein cows (565 ± 50 kg of BW, 165 ± 45 DIM at trial initiation) were

randomly assigned to a replicated 2 × 3 change over design with 14 d periods; the first 11 d of each period were for diet adaption followed by 3 d for sampling and data collection. Primiparious animals were used due to comparative milk production, DIM, and body mass. At the time of trial inisiation, there were not enough comparable multiparous cows available in the herd. Table 7.1 indicates the assignment of animals to treatment and period.

Table 7.1. Experimental design and assignment of cows to treatment and period.

		Period	
		1	2
Treatment	C	Cow 1	Cow 4
	C	Cow 2	Cow 5
	C	Cow 3	Cow 6
	BP	Cow 4	Cow 1
	BP	Cow 5	Cow 2
	BP	Cow 6	Cow 3

All experimental procedures were done from August 2016 to September 2016 at the University of Stellenbosch Welgevallen Experimental Farm, Stellenbosch, South Africa. Experimental diets contained Bioprotect treated or H_2O treated maize, a commercial HPC, molasses meal, wheat straw and lucerne hay (Table 7.2). Diets were optimized using AMTSpro (AMTS LLC, Groton, NY, USA) software. To eliminate variation in raw materials, the same batches of all raw materials were used throughout the trial. Diet moisture was regulated with H_2O addition to create a TMR DM of as close as possible to 550 g/kg.

Cows were housed individually in 6 × 4 m pens in a well ventilated, semi-open barn with a cement floor and milked twice daily in a parlor with yield recorded at each milking for each cow. Each cow had free access to a sand-bedded sleeping crate, a feeding trough, and fresh water via a ball-valve-controlled water bowl. Cows received a TMR once daily at 08h00 for 5% refusals with the amounts fed and refused recorded once daily during the data recording periods. Afimilk (AFIMILK, Kibbutz Afikim, Isreal) was used to determine milk volume (L) and body weight (kg) twice daily. Milk samples were

collected from each cow at each milking on the last 3 d of each period and analyzed for fat, crude protein, lactose and MUN concentrations using infrared analysis at a commercial laboratory (Milcolab, Cape Town, Western Cape, South Africa). The trial protocol was approved by the Stellenbosch University's Animal Ethics Committee (reference: SU-ACUD16-00157).

Table 7.2. Formulated ingredient and nutrient composition of the experimental diets.

Item	Formulation	C[1] (n = 6)	BP[1] (n = 6)
Ingredient, kg/tonne			
Lucerne hay[2]	297	297	297
Wheat straw	101	101	101
Commercial Molasses meal[3]	14	14	14
Hammer milled maize	385	385	385
Commercial HPC[4]	203	203	203
Total	1000	1000	1000
Nutrient (DM)			
DM (g/kg)	528.3	559.4	571.4
Ash (g/kg)	69.5	71.9	68.1
OM (g/kg)	930.5	928.1	931.9
N (g/kg)	25.9	25.2	24.7
CP (g/kg)	161.9	157.7	154.5
EE (g/kg)	39.6	24.3	26.5
Crude fibre (g/kg)	132.1	136.5	142.3
NFE (g/kg)	125.2	169.0	180.0
NDF (g/kg)	280.5	285.6	297.0
Starch (g/kg)	317.9	377.2	373.7

[1] Treatments were: water treated at 10 L/tonne of maize (C) and Bioprotect treated at 10 L/tonne of maize (BP).
[2] DM = 87%, CP = 20.11, NDF = 36.23%.
[3] 75% sugar cane molasses + 25% sugar cane bagasse.
[4] DM = 894 g/kg, CP = 329.2 g/kg, SP = 26.8 % of CP, NDF = 239 g/kg.

7.3.3.1 Diet sample collection and analyses

Triplicate TMR samples were sampled during each period and dried at 55°C for 72 h in a forced-air oven to determine DM content (Hall and Mertens, 2008) and to prepare samples for proximate analysis. The DM content was also calculated from split TMR samples that were dried at 105°C for 72 h. Samples dried at 55°C were subsequently ground through a 1 mm screen (Hall, 2009; Lopes *et al.,* 2009) with a laboratory

hammer mill (Scientec RSA Hammer mill Ser. Nr 372; Centrotec). All TMR samples were analyzed according to AOAC (2005) procedures for CP (AOAC, 1995), ether extract (EE), crude fibre (CF) and NDF (Ankom^{220} Fiber Analyzer, ANKOM Technology, Fairport, NY, USA). The OM content (after ashing at 500°C for 6 h) was calculated as 100 – ash content. The nitrogen free extract (NFE) content of the TMR was determined by 100 - (CP + EE + moisture + ash + CF). The starch content of the TMR was determined according to the method described by Hall (2009) in Chapter 3.

7.3.3.2 Faecal sample collection and analyses

In each period, faecal grab samples were collected from each cow, twice daily over three days, to cover 04h00, 08h00, 12h00, 16h00, 20h00, and 24h00 time points over each 3 d sampling period as recommended by Lopes *et al.* (2009). Faecal samples were dried at 55°C for 72 h and ground as described previously and composited by cow within period. Composite faecal samples were analysed for DM, NDF, and starch as described previously.

7.3.3.3 Total tract nutrient digestibility

In vivo apparent total tract starch digestibility was determined using 120 h iNDF as an internal marker as described by Waller *et al.* (1980) and Lopes *et al.* (2009). All composite faecal and TMR samples were weighed in triplicate (300 +/- 10 µg) and heat-sealed in fiber filter bags (Ankom F57) of 25 µm porosity. Thereafter all sealed samples were incubated *in vitro* in buffered rumen liquid for 120 h according to the procedure of Goering and Van Soest (1970) and Van Soest and Robertson (1991). After incubation, all bags were washed in cold water for 3 cycles in a commercial twin tub washing machine (Cherney *et al.,* 1990), dried at 60°C for 72 h, and weighed to determine the amount of DM remaining in the bags. The NDF content of the bag residues (Amok, 2016) was then determined using α-amylase and sodium sulfite (Na_2SO_3) as described by Van Soest *et al.* (1991) with an Ankom^{220} Fiber Analyzer (Ankom, 2016).

Apparent total tract DM, starch and nitrogen (N) digestibilities were subsequently calculated using the following equation (Khan *et al.,* 2003; Schalla *et al.,* 2012):

Apparent total tract nutrient digestibility (%) =

100 – {100 × (TMR iNDF/faecal iNDF) × [faecal nutrient content (% of DM)/TMR nutrient content (% of DM)]}.

7.3.3.4 Production and efficiency calculations

Allen (1997) summarized results from several trials to show a relationship between ruminal pH and milk fat concentration (r^2 = 0.39). Although the effect of SARA on milk protein percentage is largely unknown, the association between ruminal pH and milk fat percentage is, however, well documented (Allen, 1997; Norlund *et al.,* 2004; Oetzel, 2004). According to Norlund *et al.* (2004) ruminal pH can thus be estimated by the following equation:

Ruminal pH = 4.44 + (0.46 × milk fat %).

As intact cows were used (available cannulated cows were not comparable) in the current trial, ruminal pH could not be measured directly, hence the use of the above formula to estimate pH.

In this study both diets contained equal amounts of energy, protein and roughage (Table 7.2). Both diets further contained raw materials of similar quality. All apparent changes in milk fat concentration in relation to milk protein concentration could therefore be attributed to a function of ruminal starch fermentation. The P:F ratio was thus calculated to be used as an indicator of SARA.

Milk crude protein, as recorded in this study, was converted to true milk protein with a factor of 0.96 (DePeters and Ferguson, 1992).

Energy corrected milk (ECM) was calculated as (NRC, 2001; Schalla *et al.,* 2012):

ECM = (0.327 × kg of milk) + (12.95 × kg of fat) + (7.2 × kg of true protein).

Fat corrected milk (4% FCM) was calculated as (NRC, 2001):

FCM (4%) = (0.4 × kg of milk) + {15 × (Milk fat (%) / 100) × kg of milk}.

7.4 Statistical analysis

Results were analyzed using REML to ensure consistency of *P* values on VEPAC of STATISTICA version 13 (Stat Soft, Inc., Tulsa, USA). Carryover effects were tested as interaction between treatment and sequence. Significance was declared at $P \leq 0.05$ and tendencies at $P \leq 0.10$.

7.5 Results and Discussion

In a study evaluating particle size of dry milled maize (4.8, 2.6 and 1.2 mm), Callison *et al*. (2001) reported that the fine grinding of maize greatly increased ruminal starch fermentation. Reducing the particle size of maize affected true ruminal fermentation of NSC quadratically (49.8, 46.5, and 87.0%, respectively) (Callison *et al*., 2001). Knowlton *et al*. (1998) further showed that TTSD increased when dry maize mean particle size is decreased. Results of Allen *et al.* (2008), investigating high and low vitreous maize and mean particle size, indicated that TTSD of dairy cows was higher for low vitreous maize compared to high, but was unaffected by fineness of grind. This suggests that maize vitreousness has a greater influence on TTSD compared to fineness of grind.

The reason for relatively short periods (14 d) used in this study was the fact that the maize was pre-sourced from a local producer and a limited quantity was available. However, the exact period intervals were shown to be sufficient in a Journal of Dairy Science article by Lopes *et al.* (2009).

According to literature *in vivo* digestibility results could be influenced by a variety of factors including:

- Particle size (Shaver *et al.,* 1986; Woodford and Murphy, 1988; Bal *et al.,* 2000)
- Starch content (Burroughs *et al.,* 1949; Chappell and Fontenot, 1968; Grant and Mertens, 1992; Visser *et al.,* 1998)
- Ruminal starch degradability (Cooke and Bernard, 2005)
- pH (Grant and Mertens, 1992)
- Passage rate (Oba and Allen, 1999)
- Parity (Keuhn *et al.,* 1999)
- Stage of lactation (Keuhn *et al.,* 1999).

It is therefore clear that *in vivo* digestibility results could be influenced by multiple factors and total tract digestibility results are often multifactorial influenced.

The composition and analysis of the experimental diets are presented in Table 7.2. Differences in observed diet DM could be attributed to accuracy of on farm mixing. Despite minimal differences between experimental diets, both the control (C) and treatment (BP) diets differed in CP, EE and starch compared to the formulated diet. Trial diets were formulated one month prior to the mixing of the diets. The differences between actual vs. formulated diets could be explained by the use of a commercial HPC, which would be subjected to normal commercial industry monthly minimum cost reformulation. As NFE is affected by CP, EE, moisture, ash and CF differences between the formulated and trial diets can by explained by the differences in CP and EE. Mean averages between C and BP diets, however, were minimal; therefore the variation between the formulated and trial diets was ignored.

No significant interaction between treatments and sequence of treatment allocation was observed with any of the recorded variables, therefore any carryover effects between treatments could be excluded. As the trial diets only differed in terms of maize treatment, this was to be expected. The amounts and ratios of all raw materials in the trial diets were exactly the same (Table 7.2), therefore the RMO's would be well adapted to the specific diets resulting in little or no adaptation, explaining the absence of any carryover effects. Furthermore, diets were very close to the normal farm diet, meaning that there was no need for a long adaptation period.

The effect of the starch binder treatment of low vitreous maize on total tract digestibility

parameters is presented in Table 7.3. The 120 h iNDF was used as an internal marker to calculate *in vivo* apparent digestibility values.

Table 7.3. Effect of Bioprotect treatment of low vitreous maize on least squares means for apparent total tract nutrient digestibilities[1,2]

	Treatment[1,2]			
Item	C	BP	SEM	*P*
	--% Digestibility--			
DM	55.84	55.66	1.04	0.82
Starch	94.47	91.47	0.88	0.05
N	51.20	51.26	2.27	0.98

[1] Maize treated with water at 10 L/tonne grain (C) and maize treated with Bioprotect at 10 L/tonne grain (BP) fed in TMR.
[2] Determined using 120 h indigestible NDF (iNDF) as internal marker.

Least square means for apparent total tract dry matter (DM) and nitrogen (N) digestibilities did not differ between treatments (Table 7.3). These results are in agreement with Lopes *et al.* (2009) who investigated apparent total tract digestibilities of crude protein (CP) and DM when maize of different vitreousness were fed to lactating dairy cows. The rate of ruminal starch degradation of maize (k_d) of different vitreousness has been well documented and it was found that vitreousness is negatively correlated with the rate of starch digestion (Philippeau and Michalet-Doreau, 1997; Correa *et al*., 2002; Ngonyamo-Majee *et al*., 2008ab). Fractional rate of and extent of ruminal starch disappearance results from previously in this dissertation (Chapter 4) also confirmed the reduction. In the current study, the hypothesis was that the binding of some starch in the rumen would not affect apparent total tract digestibility of CP and DM, similar to what has been reported for vitreousness. It could therefore be concluded that apparent total tract digestibility of both DM and CP was not affected by ruminal rate or extent of starch digestion. Rumen undegradable protein (UDP) is defined as that portion of dietary protein that escapes degradation by ruminal microorganisms and is passed into the small intestine for digestion and absorption (Hersom and Carter, 2017). If the binder did bind some protein in the rumen to create a higher proportion of UDP, it did not manifest in a higher apparent total tract CP digestibility. In this trial, binder treatment was actually applied to the starch component (maize) prior to TMR mixing and thus likely had no effect on the main protein carrying

raw materials. The CP contribution of the grain was most likely too low to achieve any considerable ruminal binding of dietary protein and therefore had no effect on total tract CP, N or DM digestibility.

Apparent total tract starch digestibility (TTSD) was significantly ($P \leq 0.05$) lower for BP compared to C. (Table 7.3). This was not expected, as the hypothesis was that the presumably bound starch (as estabilised by *in vitro* disappearance results of Chapter 6) would be released at a low pH in the duodenum, thus improving TTSD rather than decreasing it. The TTSD results of this study are in contrast to results of Gonzalez *et al.* (2014). The latter authors reported similar TTSD values in a trial when starch binder treated wheat was fed to sheep. The reasons for the contradicting results are not readily explicable. It should be taken into account that Gonzalez *et al.* (2014) used wheat, which has a significantly higher fermentation rate than even low vitreous maize (Moharrery *et al.,* 2013; Opatpatanakit *et al.*, 1994; Herrera-Saldana *et al.,* 1990). Species could also have an effect; dairy cows were used in the current trial, while Gonzalez *et al.* (2014) used sheep. Sheep are known to be more efficient to digest starch than cows, both ruminally and post ruminally (Morrison, 1959; Nocek and Tamminga, 1991; Rowe *et al*., 1999).

With in *situ* data, Lykos and Varga (1995) showed a linear inverse relationship between particle size obtained after processing and ruminal starch digestibility of maize. This was also confirmed with results of Chapter 6 of this dissertation. Callison *et al*. (2001), in support, reported increased ruminal starch degradability as well as marginally increased TTSD when maize particle size was reduced (4.8 vs. 1.2 mm grind size). Because the rate of ruminal fermentation of wheat compared to maize is significantly higher (Herrera-Saldana *et al.,* 1990), differences in the rate of starch degradability between wheat and maize, in combination with a 4 mm grind size, could possibly also explain some of the TTSD differences observed between the current study and documented studies. Differences in amylopectin and amylose are responsible for differences in the rate and extent of ruminal starch disappearance between wheat and maize (McAllister *et al.,* 1993; Opatpatanakit *et al.,* 1994; Moharrery *et al.,* 2013). The differences in disruption of amylopectin and amylose by milling could also have impacted on the differences observed by Dunshea *et al.* (2012ab) for wheat and those observed in the current study for maize.

Earlier in this dissertation (Chapter 6) it was shown that Bioprotect treatment of the same maize ground through a 4 mm sieve reduced ($P < 0.05$) *in vitro* fractional rate and extent of starch disappearance compared to a distilled water treatment. As the

exact same maize was used in the cow trial, it can be accepted with a reasonable degree of certainty that BP also reduced ruminal starch degradation in the cows used in the current trial. As TTSD in this study decreased ($P < 0.05$) with BP treatment compared to C, the differences between treatments may be explained by the hypothesis that the starch had been effectively bound to prevent some degradation in the rumen but that the complexes did not decompose effectively under the more acidic conditions of the small intestine to expose it for further digestion.

The faecal starch content of the different treatments in the current study showed a tendency ($P < 0.09$) to be higher for BP compared to C. Mean values were 72 g/kg for BP and 45 g/kg for C. Gonzalez *et al.* (2014), in contrast, reported significantly lower faecal starch values with Bioprotect treated vs. untreated wheat. Although faecal starch content alone, without quantifying faecal output, is not an accurate determinant of digestibility, it remains an important vector for TTSD calculation and is merely another observation regarding the lower TTSD values observed in the BP treatment. In a literature review, Owens and Zinn (2005) reported a high correlation ($r^2 = 0.94$) between faecal starch content and TTSD. Ferraretto and Shaver (2012) and Fredin *et al.* (2014) reported a similar correlation ($r^2 = 0.94$) between faecal starch content and TTSD and concluded that feacal starch concentration could be used to monitor TTSD. Results of the current study showed similar relationships as observed by Fredin *et al.* (2014) and the data fitted the equation (TTSD = 100-(1.25 x % feacal starch)) published by Fredin *et al*. (2014) to accurately predict TTSD from feacal starch. Using results of a field study with 32 commercial herds, Powel-Smith *et al.* (2015) also reported that TTSD exceeds 95% and is closely related to feacal starch concentration ($r^2 = 0.98$). Although TTSD in this study was slightly lower than reported by the latter authors, feacal starch was also closely related to TTSD. Despite species, grain type or treatment differences, this study confirmed that TTSD in ruminants is at least 90% (Tucker *et al*., 1968; Ørskov, 1986; Herrera-Saldana *et al.,* 1990; Gonzalez *et al.,* 2014).

Least squares mean values of DMI, milk yield and feed efficiency of lactating dairy cows with the treatment of low vitreous maize with a starch binder are presented in Table 7.4. No significant differences or tendencies were found in either of the production parameters or feed conversion ratios between treatments. Average DMI values were almost identical, indicating no difference in palatability. Most previous work documented in the literature with the use of the same starch binder was mainly focused on *in vitro* studies, omitting intake and palatability measurements (Dunshea

et al., 2012ab). However, with an *in vivo* study evaluating the effect of the same starch binder treatment of wheat with sheep, Gonzalez *et al.* (2014) also did not indicate any differences in palatability. The influence of DMI on production or total tract digestibility parameters could therefore be excluded.

Table 7.4. Effect of Bioprotect treatment of low vitreous maize on DMI, milk yield and feed efficiency of lactating dairy cows.

	Treatment[1]			
Item	C	BP	SEM	*P*
DMI, kg/d	23.8	23.8	0.57	0.98
Milk yield, kg/d	27.1	26.5	2.04	0.18
ECM, kg/d	26.8	26.4	1.64	0.70
4% FCM, kg/d	24.7	24.5	1.48	0.82
Feed efficiency				
FE 1[2]	1.12	1.11	0.05	0.79
FE 2[3]	1.14	1.11	0.07	0.47

[1] Maize treated with water at 10 L/tonne grain (C) and maize treated with Bioprotect at 10 L/tonne grain (BP) fed in TMR.
[2] FE determined by: kg of ECM/kg of DMI.
[3] FE determined by: kg of milk/kg of DMI.

Least squares means of average daily milk yield did not show any tendency or significant differences between treatments. The digestion of maize and the subsequent absorption of glucose is a major glucogenic driver for milk production and composition (Kittivachra *et al.,* 2007). It could therefore be argued that the amount of glucose, irrespective of site digested and absorbed, would determine production response. This is in accordance to reports of similar TTSD of starch binder treated wheat fed to sheep compared to untreated wheat (Gonzalez *et al.,* 2014).

As other production parameters, ECM and 4% FCM are functions of milk yield, they also did not show any differences with treatment. The almost identical ECM and 4% FCM observation between diets could thus be explained by the slightly numerical lower milk yield and slightly numerical higher milk fat concentration with BP compared to C. Both methods for calculation of feed efficiency (FE) did not show any significant differences between treatments. The observed lower than average absolute FE values (Lopes *et al.,* 2009) of both ECM and 4% FCM can be attributed to the higher days since calving (mean DIM = 165 ± 45). McNamara *et al.* (2008) showed that milk yield would be 14 % lower when lactating cows are milked twice compared to three times

per day. As milking in this study was done only twice daily, milk yield would be lower compared to three times, which in turn would have reduced FE. As the animals were primiparous, compensatory growth could also explain the relative low absolute FE values recorded.

The effect of treatment on milk composition and estimated ruminal pH are presented in Table 7.5. Despite slightly higher numerical least squares means for milk fat concentration (%) observed when BP was fed compared to C, differences were not significant. This result would suggest the absence of assidoses in both C and BP.

Table 7.5. Effect of Bioprotect treatment of low vitreous maize on milk composition and estimated rumen pH.

	Treatment[1]			
Item	C	BP	SEM	*P*
Milk fat, %	3.45	3.52	0.17	0.60
Milk fat, kg/d	0.92	0.92	0.05	0.95
Crude milk protein, %	3.21	3.19	0.09	0.76
Crude milk protein, kg/d	0.87	0.84	0.06	0.22
True milk protein, %	3.08	3.07	0.09	0.76
True milk protein, kg/d	0.83	0.81	0.06	0.22
MUN, mg/dL	9.11	9.5	0.47	0.58
Lactose, %	4.84	4.83	0.04	0.54
SCC (x1000 cells/mL)	186.8	170.2	38.9	0.77
P:F	0.92	0.88	0.05	0.43
Predicted ruminal pH[2]	6.03	6.06	0.08	0.60

[1] Maize treated with water at 10 L/tonne of grain (C) and maize treated with Bioprotect at 10 L/tonne of grain (BP) fed in TMR.
[2]Ruminal pH was predicted as follows: pH = 4.44 + (0.46 * milk fat %), according to Norlund *et al.* (2004).

Least squares means of total milk fat produced were the same between the treatments (Table 7.5). Yields of fat, protein, non-fat solids and total solids are highly and positively correlated with milk yield (Looper, 2014). The concentration of milk fat and milk protein, however, decreases as yield increases (Looper, 2014).

Both crude protein and true milk protein concentration in the milk were unaffected by treatment. A potential result of higher ruminal starch degradability would be higher propionate absorption into the blood stream. This would have caused increased insulin levels, which in turn should stimulate an increased uptake of protein by the mammary

gland (Rius *et al.,* 2010). The starch binder treatment of the low vitreous maize did not affect milk protein concentration, indicating equal propionate availability and portal insulin levels. Both true and crude milk protein yield did not differ significantly either. As milk protein yield is a function of milk yield and milk protein concentration, this is to be expected because neither of these parameters differed significantly.

The composition of the diet and the subsequent ruminal pH would affect milk crude protein to fat (P:F) ratio (Norlund *et al.,* 2004; Oetzel, 2004). Milk protein (%) increases linearly with increasing intake of non-structural carbohydrates (i.e. starch and sugar), provided protein intake is not limited, and, in general, milk fat (%) declines accordingly (Bargo *et al.,* 2003; Roche *et al.,* 2006). By feeding lactating dairy cows a maize grain based concentrate, Higgs *et al.* (2013) reported a P:F ratio of 0.91. Changes in milk composition and P:F ratio with alterations in diet composition are due to changes in VFA production: more propionate is produced when starch is consumed and more acetate is produced when fibre is consumed (Van Soest, 1994). Propionate absorption into the blood stream causes increased insulin levels, which stimulate an increased uptake of protein by the mammary gland (Rius *et al.,* 2010). Therefore, P:F ratio usually increases when cows are fed cereal- based concentrate feeds. In comparison, acetate is the building block for milk fat; therefore, feeding a fibrous diet, which directs rumen fermentation towards more acetate production, will increase milk fat concentration. This change in milk composition will occur irrespective of the energy status of the cow. The P:F ratio is a function of milk protein and fat concentration; therefore, the slightly lower numerical ratio with BP compared to C would be a result of the slight (albeit insignificant) difference in milk fat concentration observed, while milk protein concentration remained equal. The P:F ratio usually increases when cows are fed cereal based concentrate feeds. Least squares means values for P:F ratios (0.92 vs. 088) reported in this study were similar and in agreement with documented work where high producing dairy cows were fed a high grain TMR diet (Higgs *et al.,* 2013), and SARA was absent. Although not significant, the slightly numerical lower P:F ratio of BP compared to C, might be indicative of slightly less starch digested in the rumen. This could be explained by the use of the starch binder. Estimated ruminal pH also did not differ between treatments. At a low ruminal pH (< 6), rumen function is considered to be sub-optimal (Beauchemin *et al.,* 1999; Dehghan-banadaky *et al*., 2007). The absence of SARA is therefore supported by the estimated ruminal pH (pH > 6) with both experimental diets.

Urea, an important end product of nitrogen metabolism in dairy cows, is mostly

synthesized in the liver and transported to the kidneys for excretion via urine. The concentration of urea in the blood rapidly equilibrates with other body fluids, including milk (Gustafsson and Palmquist, 1993). In a study by Eicher *et al.* (1999), evaluating factors that affect MUN in dairy herds, the daily amount of rumen soluble protein fed was positively related with MUN in 70% of herd models. Considerable variation among herds could influence the relationships between MUN and milk protein content and is affected by several factors: parity, daily milk yield, and DIM (Eicher *et al.,* 1999). The MUN content can thus be used as an indicator of the adequacy of protein and the balance between energy and protein in lactating dairy cow diets (Broderick and Clayton, 1997; Wattiaux and Karg, 2004). Values of between 8-10 mg/dL for MUN are considered to be slightly low (Ishler, 2016). The slightly low absolute MUN values recorded in this study (Table 7.5) are related to the lower CP and higher starch contents in the actual experimental diets compared to the formulated diet. In this study no significant differences were, however, found with regards to MUN means between treatments.

In a trial with 148 Holstein dairy cows Henao-Velásquez *et al.* (2014) reported that milk lactose concentration varied between 3.77% and 5.11% with a median of 4.51%. Lactose concentrations also increased as DIM increased (Henao-Velásquez *et al.,* 2014). Miglior *et al.* (2007) and Ptak *et al.* (2012) in contrast reported that the lactose curve was very similar to the milk yield curve, with a maximum value between 30 and 60 days and a gradual decrease in the remaining days. Lactose concentrations of both experimental diets were above average (Henao-Velásquez *et al.,* 2014), indicating healthy animals with minimal stress levels during the trial period. As lactose concentrations tend to increase with DIM, the relatively high lactose values observed in the current trial (Table 7.4) might also be related to the longer DIM of the trial animals (Henao-Velásquez *et al.,* 2014). Henao-Velásquez *et al.* (2014) further showed a negative lactose relationship with respect to transformed-SCC milk. Miglior *et al.* (2006) found similar results in cows that had low lactose concentrations when SCC levels increased. Miglior *et al.* (2007) reported a negative correlation (r^2 = -0.20) between lactose and SCC, while lactose was not correlated (P = 0.096) with milk yield (Miglior *et al.,* 2007). In the current study, almost identical lactose means were observed for C and PB, suggesting that one might expect similar SSC levels. In Table 7.5 it can be seen that SCC did indeed not differ between treatments. Normally, in milk from a healthy mammary gland, the SCC is lower than 1×10^5 cells/mL, while bacterial infection can cause an increase to above 1×10^6 cells/mL (Sharma *et al.,* 2011). The relatively low SCC mean values observed in the current study (Table 7.5) are an

indication that the animals experienced low levels of stress and infection during the trial period.

7.6 Conclusion

The starch binder treatment of maize ground to pass a 4 mm hammer mill screen and mixed into a TMR for lactating Holstein cows, did not alter milk yield or milk composition. Apparent total tract CP and DM digestibilities were not affected by treatment, but treating low vitreous maize with the starch binder decreased total tract starch digestibility (TTSD). Despite species, grain type or treatment differences, this study confirmed that TTSD in ruminants is at least 90%. The current study further provides preliminary evidence that the treatment of maize (milled through a 4 mm screen) with a commercial starch binder may decrease TTSD in dairy cows. The results suggested that the protection of maize starch might not allow the treated particles to be digested in the lower intestine. If the aim of the starch binder treatment is to increase total tract starch digestion, the results from the current study showed the opposite and suggest that no additional income could be generated to cover treatment costs and that it would produce a negative return on investment. If, on the other hand, the objective of the starch binder treatment is to slow starch fermentation in the rumen when high levels of low vitreous maize is fed and thereby preventing the risk of SARA or acute acidosis, then it would indeed have merit. More research is required to test the effect of Bioprotect on the ruminal health of high yielding dairy cows that receive high levels of low vitreous maize.

7.7 References

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CHAPTER 8

Conclusion and recommendations

8.1 Conclusion

Global climatic changes demand the continuous genetic improvement of maize hybrids to meet the higher crop yield requirements to match the ever-increasing consumer demand. Often, an unintended consequence of genetic improvement results in increased maize vitreousness or hardness. High vitreousness is often a restrictive obstacle for ruminant animal performance. There is thus a requirement for a rapid, simple and inexpensive method to accurately determine maize vitreousness on a regular basis on site for application in the animal feed industry. All the methods investigated in the current study, namely PSI, NIR (at a single wavelength of 2230 nm absorbance), XCT, RVAPV and RVAPT were found to be accurate to determine maize hardness. However, the only methods that can be applied in the animal feed industry in practice are PSI and NIR. As NIR technology is already available and widely used at most feed mills, it was concluded that NIR analysis at a single absorbance wavelength of 2230 nm meets the requirements to determine maize vitreousness in the animal feed industry.

In vitro disappearance results of the current study confirm an increased rate and extent of ruminal starch disappearance as maize vitreousness decreases. Results further provide evidence that significant inverse linear and quadratic relationships exist between NIR hardness index values at a single absorbance wavelength of 2230 nm on the one side and k_d and PRD responses on the other side. The use of NIR technology to determine rapid, inexpensive k_d and PRD predictions of maize without the use of time consuming, expensive *in vitro* analyses could enable the animal feed industry to make timeous decisions regarding batch delivery of maize and to formulate diets more accurately. More precise rumen kinetical parameters, as required by modern mechanistic and dynamic models, can therefore be rapidly determined by NIR.

Maize as a major provider of glucose to ruminants is fermented in the rumen to mainly propionate, which in turn is a major precursor for gluconeogenesis in the liver. Portal glucose from the liver would ultimately lead to milk production via lactose.

However, the rate and extent of maize (especially types with a very low vitreousness) degraded in the rumen might overwhelm the buffering capacity of the rumen and lead to acidosis. The use of high amounts and/or highly fermentable carbohydrates such as highly processed grains are more than often required to sustain animal production, as in the case of high yielding dairy cows. Therefore, the treatment of maize with a commercial starch binder in an effort to shift some digestion of the dietary starch to the lower intestines could be beneficial in high producing ruminants. Sites of digestion were, however, not determined in the current study. Both ruminal *in vitro* gas production and *in vitro* starch disappearance values were, irrespective of treatment, shown to be faster and more extensive for soft maize compared to hard maize. In the current study, rate and extent of *in vitro* maize starch disappearance were not affected by the binder at a 1 mm grind size.

Bioprotect treatment of the 4 mm ground low vitreous maize, however, indicated decreased *in vitro* fractional rate of disappearance (k_d) and predicted ruminal degradability (PRD). When high amounts of low vitreous maize are fed, results of the current study suggest that the associated risk of metabolic acidosis could be decreased by treatment with a starch binder (10 L/tonne grain) and processing through a 4 mm screen.

Results of the current study also indicated that both the rate and extent of ruminal disappearance of low vitreous maize are significantly higher when particle size is reduced by milling through a 1 mm screen compared to 4 mm. By reducing maize particle size, the surface area available for microbial attack is increased, and therefore enhances the rate and extent of ruminal degradation.

Starch binder treatment of low vitreous maize did not change *in vivo* milk yield, milk composition, estimated ruminal pH or feed efficiency of lactating Holstein cows compared to the control treatment. Further digestibility studies may shed more light on the inter-relationships between the mentioned factors.

Application of a commercial starch binder to a 4 mm grind size low vitreous maize did not affect apparent total tract crude protein or DM digestibility. However, treatment with the starch binder decreased apparent total tract starch digestibility significantly (P = 0.05). The explanation for the observed lower apparent TTSD of BP compared to C is not simplistic. Without doubt a multi-factorial combination of factors had an impact on apparent TTSD as observed in this study. Starch digestion in the total digestive tract of cows was, irrespective of treatment, shown to be at least 90% (91.5% for the

treatment and 94.5% for the control). As no additional income could be generated to cover treatment cost, a negative return on investment may be expected if increased utilization efficiency of starch is the aim. However, unchanged production parameters with the use of less net total tract starch and the subsequent financial benefits may warrant more research, especially in terms of decreasing the risk of acute acidosis or SARA.

8.2 Recommendations

Maize vitreousness prediction with the aid of NIR technology, using a single absorbance wavelength of 2230 nm, was found to be the most practical method for routine analysis in the animal feed industry. Results of this study suggest that NIR technology require accurate maize hardness calibrations. An inverse relationship between NIR hardness index values and k_d and PRD was determined, but only 1 mm maize was used in that part of the study. As grind size affects ruminal starch disappearance kinetics, the impact of processing would need to be established. Accurate k_d and PRD predictions of different processed maize (type and extent of processing) would not only enable field nutritionists to do more accurate ruminant model predictions and formulations, but would also ensure optimal use of maize within the animal feed industry.

Further *in vitro* research is also required to determine if the same inverse relationships (as with maize in the current study) or any other relationships exist between NIR hardness index values and fractional rate and extent of starch disappearance of different grain species.

Breakeven and sensitivity analysis should be used to determine the relative predicted animal production value of maize with different vitreousness indices compared to cost. The use of accurate k_d and PRD calibrations from rapid NIR hardness index analysis will enable the animal feed industry to optimize maize inclusion and formulation, hence the optimal utilization and costing of maize of different vitreousness for ruminant application.

8.3 Future research

Further *in vitro* and *in vivo* digestibility studies are therefore required to confirm the results of the current study. The impact of and the inter-relationships of factors that could affect TTSD of starch binder protected grain that warrant further investigation can be summarized as follows:

- Ruminal pH
- Small intestinal pH
- Small intestinal enzymatic availability and activity
- Dosing rate
- Grain vitreousness
- Protein content in the diet
- Grain particle size
- Grain specie differences
- Animal specie differences

The risk of ruminal assidoses associated with the use of high quantities of highly fermentable carbohydrates would require continuous research efforts to alleviate a reduction in ruminal pH without decreasing animal performance.

Appendix 1

Sample No.	PSI (106 µm) Soft (%)	PSI (106 µm) Hard %	NIR (2230 nm) Hardness index	Climate	Cultivation	Origin	Colour
1	30.2	69.8	12.41	Cold Semi-arid	Dry land	North-West	Yellow
2	34.1	65.9	11.75	Cold Semi-arid	Dry land	North-West	Yellow
3	37.5	62.5	8.44	Cold Semi-arid	Dry land	Gauteng	Yellow
4	41.8	58.2	7.14	Cold Semi-arid	Dry land	North-West	Yellow
5	35.5	64.5	6.55	Irrigated (cold desert)	Irrigation	Vaalharts	Yellow
6	36.6	63.4	6.72	Cold Semi-arid	Dry land	North-West	Yellow
7	39.0	61.0	7.24	Irrigated (cold desert)	Irrigation	Vaalharts	Yellow
8	35.1	64.9	9.98	Cold Semi-arid	Dry land	North-West	Yellow
9	37.8	62.2	9.19	Cold Semi-arid	Dry land	North-West	Yellow
10	43.3	56.7	3.94	Humid Subtropical	Dry land	Gauteng	Yellow
11	38.0	62.0	5.99	Cold Semi-arid	Dry land	North-West	White
12	37.7	62.3	6.82	Cold Semi-arid	Dry land	North-West	Yellow
13	39.8	60.2	6.95	Humid Subtropical	Dry land	Limpopo	Yellow
14	39.4	60.6	7.14	Cold Semi-arid	Dry land	Gauteng	Yellow
15	36.5	63.5	8.22	Cold Semi-arid	Dry land	North-West	Yellow
16	38.1	61.9	6.21	Irrigated (cold desert)	Irrigation	Lower Orange	Yellow
17	37.3	62.7	8.31	Cold Semi-arid	Dry land	Free State	Yellow
18	39.7	60.3	7.21	Humid Subtropical	Dry land	Limpopo	White
19	36.0	64.0	8.53	Cold Semi-arid	Dry land	North-West	Yellow
20	48.9	51.1	1.59	Humid Subtropical	Dry land	Limpopo	Yellow
21	38.5	61.5	7.75	Humid Subtropical	Dry land	Limpopo	Yellow
22	40.5	59.5	6.20	Cold Semi-arid	Dry land	Free State	Yellow
23	39.8	60.2	6.15	Irrigated (cold desert)	Irrigation	Vaalharts	Yellow
24	37.7	62.3	6.59	Irrigated (cold desert)	Irrigation	Lower Orange	Yellow

25	41.4	58.6	4.61	Cold Semi-arid	Dry land	Gauteng	Yellow
26	34.5	65.5	8.98	Cold Semi-arid	Dry land	Gauteng	Yellow
27	35.0	65.0	6.04	Cold Semi-arid	Dry land	North-West	Yellow
28	37.8	62.2	6.87	Irrigated (cold desert)	Irrigation	Vaalharts (Jan Kempdorp)	Yellow
29	37.6	62.4	7.54	Irrigated (cold desert)	Irrigation	Vaalharts (Jan Kempdorp)	Yellow
30	38.3	61.7	7.38	Humid Subtropical	Dry land	KZN	Yellow
31	41.4	58.6	4.04	Unknown	Unknown	Ukraine (Ship 1)	Yellow
32	40.0	60.0	6.39	Irrigated (cold desert)	Irrigation	Vaalharts	Yellow
33	37.1	62.9	6.38	Cold Semi-arid	Dry land	Free State	Yellow
34	39.5	60.5	5.34	Cold Semi-arid	Dry land	Gauteng	Yellow
35	42.1	57.9	2.99	Unknown	Unknown	Ukraine (Ship 1)	Yellow
36	40.6	59.4	5.28	Irrigated (cold desert)	Irrigation	Vaalharts	Yellow
37	39.9	60.1	5.26	Irrigated (cold desert)	Irrigation	Vaalharts	Yellow
38	37.8	62.2	7.34	Irrigated (cold desert)	Irrigation	Vaalharts	Yellow
39	38.8	61.2	6.89	Cold Semi-arid	Dry land	Gauteng	Yellow
40	37.7	62.3	7.31	Cold Semi-arid	Dry land	Gauteng	Yellow
41	36.3	63.7	7.33	Cold Semi-arid	Dry land	Highveld	Yellow
42	36.9	63.1	7.76	Cold Semi-arid	Dry land	Gauteng	Yellow
43	39.4	60.6	5.20	Irrigated (cold desert)	Irrigation	Vaalharts	Yellow
44	40.2	59.8	7.52	Cold Semi-arid	Dry land	Free State	Yellow
45	38.6	61.4	6.93	Irrigated (cold desert)	Irrigation	Lower Orange	Yellow
46	39.2	60.8	7.82	Cold Semi-arid	Dry land	Gauteng	Yellow
47	38.5	61.5	8.50	Cold Semi-arid	Dry land	Free State	Yellow
48	41.9	58.1	5.47	Cold Semi-arid	Dry land	Gauteng	White
49	44.7	55.3	4.84	Cold Semi-arid	Dry land	Gauteng	White
50	46.0	54.0	2.81	Unknown	Unknown	Ukraine (Ship 2)	Yellow
51	45.8	54.2	3.83	Unknown	Unknown	Ukraine (Ship 2)	Yellow
52	45.0	55.0	3.25	Unknown	Unknown	Ukraine (Ship 2)	Yellow

53	39.0	61.0	5.78	Cold Semi-arid	Dry land	Free State	Yellow
54	49.3	50.7	2.33	Humid Subtropical	Dry land	Limpopo	Yellow
55	43.6	56.4	5.44	Humid Subtropical	Dry land	Limpopo	Yellow
56	36.9	63.1	8.52	Cold Semi-arid	Dry land	Highveld	Yellow
57	38.9	61.1	7.78	Cold Semi-arid	Dry land	Highveld	Yellow
58	43.9	56.1	6.23	Humid Subtropical	Dry land	KZN	Yellow
59	39.9	60.1	7.44	Humid Subtropical	Dry land	KZN	Yellow
60	45.2	54.8	6.17	Humid Subtropical	Dry land	KZN	Yellow
61	35.6	64.4	8.93	Cold Semi-arid	Dry land	Highveld	White
62	32.8	67.2	5.98	Cold Semi-arid	Dry land	Free State	White
63	34.1	65.9	7.78	Humid Subtropical	Dry land	KZN (Dundee)	Yellow
64	37.2	62.8	5.27	Cold Semi-arid	Dry land	Highveld (Holmdene)	Yellow
65	36.7	63.3	5.78	Cold Semi-arid	Dry land	Highveld (Holmdene)	Yellow
66	31.2	68.8	8.73	Cold Semi-arid	Dry land	Highveld (Holmdene)	Yellow
67	36.8	63.2	5.97	Cold Semi-arid	Dry land	Highveld	Yellow
68	40.7	59.3	4.34	Humid Subtropical	Dry land	KZN	Yellow
69	36.2	63.8	7.39	Cold Semi-arid	Dry land	Highveld	Yellow
70	40.7	59.3	3.86	Humid Subtropical	Dry land	KZN	Yellow
71	41.8	58.2	6.55	Cold Semi-arid	Dry land	Highveld	Yellow
72	41.1	58.9	5.99	Cold Semi-arid	Dry land	Highveld (Holmdene)	Yellow
73	39.7	60.3	8.41	Cold Semi-arid	Dry land	KZN (Vryheid)	Yellow
74	43.4	56.6	5.08	Cold Semi-arid	Dry land	Highveld (Holmdene)	Yellow
75	45.0	55.0	4.32	Humid Subtropical	Dry land	KZN (Dunhauser)	Yellow
76	40.3	59.7	6.94	Cold Semi-arid	Dry land	KZN (Vryheid)	White
77	39.0	61.0	8.86	Irrigated (cold desert)	Irrigation	Vaalharts	White
78	40.4	59.6	6.46	Cold Semi-arid	Dry land	KZN	Yellow
79	43.5	56.5	5.85	Cold Semi-arid	Dry land	Highveld (Holmdene)	Yellow
80	38.9	61.1	8.68	Cold Semi-arid	Dry land	North-West	Yellow
81	37.6	62.4	6.84	Irrigated (cold desert)	Irrigation	Vaalharts	Yellow

82	42.9	57.1	2.93	Irrigated (cold desert)	Irrigation	Vaalharts	Yellow
83	36.7	63.3	7.06	Cold Semi-arid	Dry land	North-West (Koster)	Yellow
84	37.5	62.5	4.62	Unknown	Unknown	Ukraine (Ship 2)	Yellow
85	36.8	63.2	5.01	Unknown	Unknown	Ukraine (Ship 2)	Yellow
86	22.2	77.8	23.58	Unknown	Unknown	Pop corn 1	Yellow
87	13.3	86.7	25.73	Unknown	Unknown	Pop corn 2	Yellow
88	35.1	64.9	9.50	Unknown	Unknown	Argentina (Ship 1)	Yellow
89	35.5	64.5	11.05	Unknown	Unknown	Argentina (Ship 2)	Yellow
90	32.0	68.0	8.96	Unknown	Unknown	Argentina (Ship 3)	Yellow

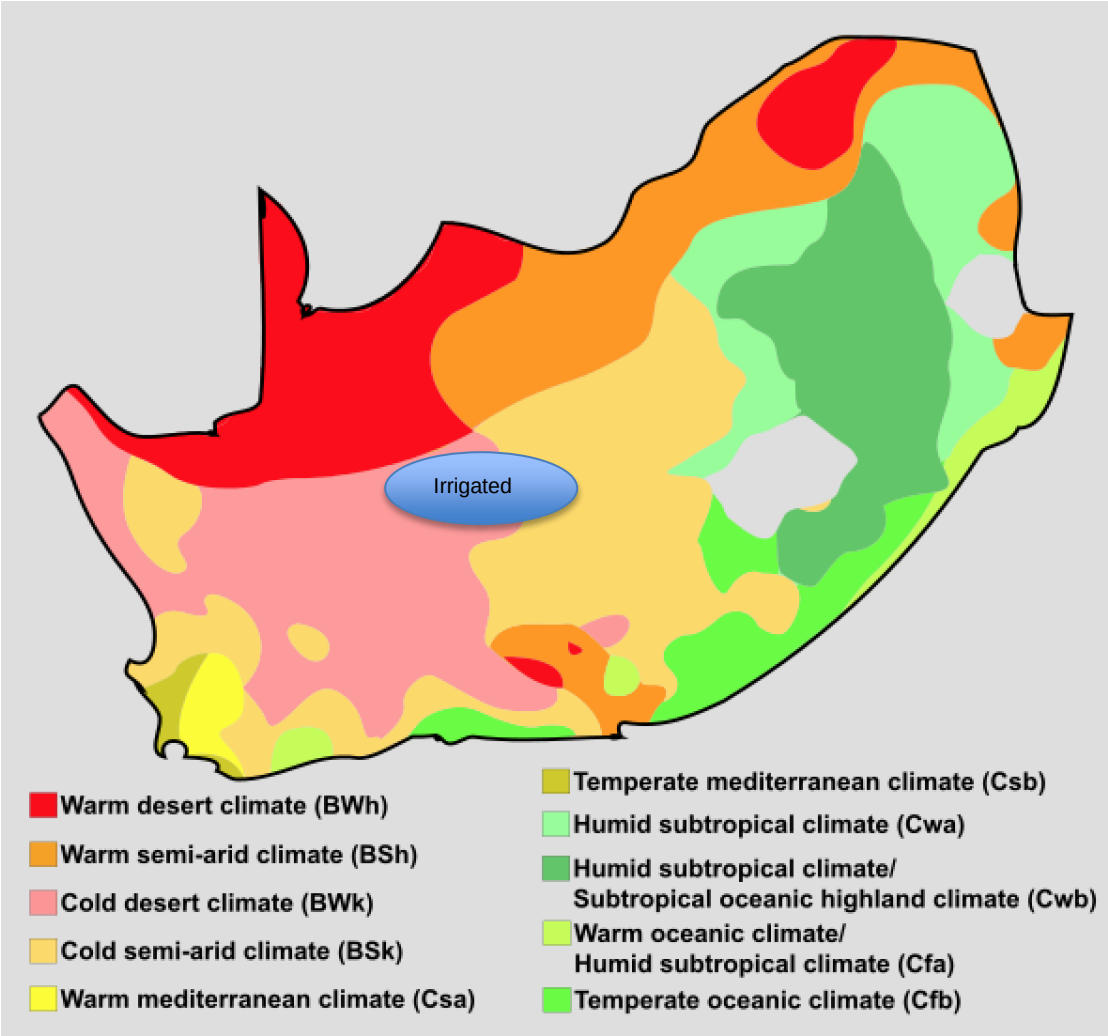
Hard selected maize samples = Red.

Soft selected maize samples = Green.

Lower Orange and Vaalharts are irrigated.

Appendix 2

The Köppen climatic classification map of South Africa (Peel *et al*., 2007).



Appendix 3

Sample allocation, treatment and incubation time for each run of the starch disappearance study in Chapter 4.

Vitreousness	Treatment[1]	Incubation time[2]	Cow
Hard	BP	6	1
Hard	BP	12	1
Hard	BP	24	1
Hard	BP	6	2
Hard	BP	12	2
Hard	BP	24	2
Hard	C	6	1
Hard	C	12	1
Hard	C	24	1
Hard	C	6	2
Hard	C	12	2
Hard	C	24	2
Soft	BP	6	1
Soft	BP	12	1
Soft	BP	24	1
Soft	BP	6	2
Soft	BP	12	2
Soft	BP	24	2
Soft	C	6	1
Soft	C	12	1
Soft	C	24	1
Soft	C	6	2
Soft	C	12	2
Soft	C	24	2
Reagent blank	None	6	1
Reagent blank	None	6	2
Reagent blank	None	12	1
Reagent blank	None	12	2
Reagent blank	None	24	1
Reagent blank	None	24	2

[1]Treatments were Bioprotect (equivalent to 10 L/tonne maize) (BP) and distilled water (equivalent to 10 L/tonne maize) (C).
[2]Hours incubated (h).

Appendix 4

Sample allocation, treatment and incubation times for each run of the grind size and Bioprotect starch disappearance study in Chapter 6.

Grind size[1]	Treatment[2]	Incubation time (h)
1	C	0
4	C	0
1	BP	0
4	BP	0
1	C	3
4	C	3
1	BP	3
4	BP	3
1	C	6
4	C	6
1	BP	6
4	BP	6
1	C	12
4	C	12
1	BP	12
4	BP	12
1	C	24
4	C	24
1	BP	24
4	BP	24
1	C	48
4	C	48
1	BP	48
4	BP	48
Reagent blank	None	0
Reagent blank	None	3
Reagent blank	None	6
Reagent blank	None	12
Reagent blank	None	24
Reagent blank	None	48

[1]Maize grind size: 1 mm (1) and 4 mm (4).
[2]Treatments were Bioprotect (equivalent to 10 L/tonne maize) (BP) and distilled water (equivalent to 10 L/tonne maize) (C).